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Abstracts of Scientific Papers

AISAL Symposium

Animal Pain Recognition and Management in Biomedical and Veterinary Research

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Main Lectures

Translational Anesthesia and Analgesia: Tools to Protect Animals while Serving Science

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The current European Animal Welfare Regulation (Directive 2010/63/UE) discusses with precision, among others, the issue of anesthesia and analgesia, providing essential points for a correct approach to the use of animals for scientific purposes. This is the legal basis, then declined by each Member country, from which the science community must begin to respond in an ever more adequate and updated way to instances of protecting welfare of animals. Although important milestones have been achieved in the field of recognition and pain control as well as in the field of anesthesia, future scenarios in biomedical research endeavor even more pressing researchers with new challenges. The acquisition of new knowledge, improved techniques and skills, and continuous updates in a transactional context will be essential tools to promote and ensure a real welfare for the animals. The transposition of techniques and protocols by human medicine (anesthesia and pain management) supported by a networking with clinicians, as well as a mutual discussion and exchange of experiences could provide suitable tools to safeguard and protect the animals, while adequately serving science. This presentation will review and illustrate, by a translational perspective, some protocols and procedures applied in preclinical and will present possible new approaches applicable for the future.

Classification of the Severity of the Experimental Procedure and the Retrospective Evaluation in the New European Directive

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The European Parliament and the Council of European Union adopted Directive 2010/63/EU governing the protection of animals used for scientific purposes. Among novelties introduced by the Directive is the request to classify all procedures on the basis of their severity, according to the criteria set out in Annex VIII. Another innovative aspect is the retrospective evaluation of severity of procedures and of any elements contributing to application of the 3Rs. During transposition of the Directive by Member States it would be desirable find a common approach to ensure consistency in its application. The main difficulty is that this type of classification is a new concept for some Member States, while others have systems already in place. In light of this, FELASA decided to set up 2 working groups on severity classification and retrospective evaluation with the objective to issue guidelines to direct the implementation and harmonization of new legislative demands with particular focus on these aspects. The objective of this presentation is to give an overview of the most innovative aspects contained in the Directive and describe activities undertaken by 2 working groups on aspects of severity classification and retrospective assessment.

Recognition of Pain in Laboratory Animals

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Scientific research has confirmed that almost all animals (included many invertebrates) possess the anatomic and physiologic features that render them able to perceive pain and not only nociception. Since laboratory animals can be submitted to painful procedures, and since the legislation protects them from pain and suffering, the assessment and treatment of pain in the various laboratory species becomes mandatory. Recognizing pain and assessing its intensity are both essential for its effec-

tive management: if pain is not recognized, then it is unlikely to be treated. While in humans self-report of pain is possible, representing the "gold standard" in the diagnosis of a painful condition, for animals, as for humans who cannot self-report (for example, the very young and those with cognitive impairment), other assessment tools are necessary. These tools should include: a presumptive diagnosis, a clinical evaluation (physical and biochemical findings), the assessment of behavioral responses to pain, the use of pain scores, and the evaluation of the response to therapy. The assessment of behavioral responses to pain is the best way to determine the degree of pain experienced by animals during both acute and persistent or chronic pain. Behavioral features related to pain in almost all laboratory animal species could be summarized in changes of posture, gait, movement, mood, appearance, facial expressions, vocalizations, and elimination habits. Operators should be trained in order to reveal also subtle changes in animal behaviors. Grimace scales have been proposed for the assessment of pain in mice, rats, and rabbits. Finally, the response to therapy represents a good index of the previous presence of pain.

Recognition and Management of Pain and Suffering in Research Studies Involving Nonhuman Primates

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Expertise and genuine consideration for animal welfare are essential to enable refinement of experimental procedures, aiming to prevent or minimize pain and suffering. Our main goal was to define and apply a system to prevent, recognize, and manage suffering during the 3 main phases of transplantation surgery: preoperative, intraoperative, and postoperative phases. In the perioperative period, the improvement of safety and efficacy of anesthesia and analgesia are critical aspects. Aiming to refine the procedure in our primate models, we implemented adjustment and refinement in the anesthetic and analgesic protocol. Indeed, a more effective management, a faster recovery, and a positive impact on animal welfare were obtained by modifying dosages of drugs administered, whilst guaranteeing the maintenance of an optimal depth of anesthesia and analgesia. Considering the postoperative phase, to recognize and manage pain and/or suffering eventually occurring, an evaluation system has been implemented, relying on the following aspects: 1) continuous clinical monitoring, ensuring the best possible approach for the prompt evaluation of the animal conditions, thus minimizing the risk of mistake or underestimation of clinical signs, 2) continuous laboratory monitoring, allowing an early recognition of any altered physiologic parameter, and 3) definition of specific assessment criteria, with regard to the termination of experimental procedures and consequent euthanasia (humane endpoint). This assessment system, based on a combined analysis of both quantitative (measurable) and qualitative (observable) elements, had useful, satisfactory, and reproducible results. We believe that our approach resulted in a reduction of animal suffering without any negative impact on scientific results.

Pain in Cephalopods: A Neglected Issue

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Cephalopods have been recently included in the Directive 2010/63/EU, the first and sole representatives among invertebrates. These animals are without doubt the phylogenetically most ancient regulated class of animals in the EU. Cephalopods belong to the phylum Mollusca represented by more than 700 extant marine-living species. The Directive covers both adults and hatchlings/juveniles (not eggs). The criteria used by legislators for the need of protection for invertebrates (that is, cephalopods in the current version) and fetuses, are based upon the scientific evidence for the sentience and capacity of invertebrate species used for experimental purposes (including fetal and embryonic forms) to "experience pain, suffering, distress or lasting harm." The assessment of the capacity to experience pain in animals is based on several issues, including: 1) possession of higher brain centers, 2) presence of nociceptors, 3) pathways connecting nociceptive system to higher brain centers, 4) evidence for opioid-like substances receptors (especially in brain), 5) modification of noxious response by analgesics, 6) behavioral responses (including associative learning). Cephalopods possess most of these characteristics; however, evidence for the presence of nociceptors and pain-regulated responses are suspected in these animals. For example, the role of pain in the modulation of behavioral responses during training and after negative reinforcement is still questioned. I will review past and current literature and present recent data on the evidence of key-genes pertaining to nociceptors family in the octopus and other cephalopod species. Using a step-by-step approach, I will also convey the most recent results and discuss future scientific endeavor required to fill this gap.

Stress and Pain in Farm Animals Used in Regulatory Veterinary Studies

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The preclinical studies for veterinary medicinal products require a constant use of farm animals whose characteristics have to meet the requirements defined in the specific EMA guidelines for each type of study. The mandatory use of animals in good health status, a proper balance between the environmental and behavioral needs of the animal and housing under controlled conditions, and, finally, the requirement to avoid drug treatments in addition to the test substance that may affect the results, represent the main issues in the welfare management of farm animals used for experimental purposes. Constant monitoring of the health status of the animals in the early stages of acclimatization and during the test management should be based on classic medical

semiotics as well as on the ability to correctly understand animal behavior as expression of a potential state of pain or stress. The knowledge of the changes in behavior as expression of pathologic conditions, environmental issues, or manipulation mistakes is fundamental to highlight the occurrence of abnormalities and take prompt action to safeguard both the results of the experimental study and the welfare of the animal.

Pain Circuitry in Animal Models

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Pain is an unpleasant sensation that is experienced when signals generated in specific peripheral receptors (nociceptors) by a (potentially) damaging stimulus reach the somatosensory cortex. Pain information reaches the spinal cord via unmyelinated or thinly myelinated central processes of small diameter nociceptors located in the dorsal root ganglia. Nociceptive signals en route to the brain are then integrated at the level of the dorsal horn of the spinal gray matter where the synapses with second order sensory neurons are located. These synapses are the target of a complex network of spinal interneurons that is mainly localized in lamina II (substantia gelatinosa) of the gray matter. Transmission/modulation of nociceptive inputs in lamina II is, in turn, mediated by an impressive number of neurotransmitters. The low molecular weight glutamate, GABA, and glycine are responsible for fast neurotransmission and likely involved in rapid adaptive responses to noxious stimuli. The high molecular weight peptides, instead, produce slower and long lasting responses that may drastically influence chronic pain. In addition, recent work has unraveled the circuitry made by a subpopulation of nociceptors expressing the growth factors BDNF and GDNF. The latter appears to be specifically responsible for a cross-talk between peptidergic and nonpeptidergic nociceptive primary afferents under inflammatory conditions. Much of the progress in our comprehension of neuronal circuitry and neurotransmission in mammalian substantia gelatinosa comes from histologic and functional studies on animal models that will be here discussed also on comparative bases.

Pain models in Biomedical Research

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The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or is described in terms of such damage." During the past years, many efforts have been paid to develop novel drugs directed towards new targets and/or to investigate the analgesic efficacy of known drugs. However, ongoing research needs better knowledge and tools to translate the basic science information into clinically effective analgesic compounds. Tissue insult may generate an acute painful effect, associated with early neural and cellular responses aimed at removing harmful stimuli, thereby limiting further injury, and favoring repair. Unlike the inflammatory pain, neuropathic pain is a chronic disease resulting from a dysfunction of the nervous system frequently due to peripheral nerve injury. Although massive effort has been devoted to investigate the complex molecular and cellular mechanisms as well as the system(s) organization of nociception involved in normal sensory and pain perception in both acute and chronic pain conditions, much still remains to be understood. From this perspective, the management of pain in both acute and chronic conditions remains far from an optimal resolution. In this context, different experimental pain models have been established as cost-effective tools for assessing the mechanisms underlying the nociceptive process and to address the research for new antiinflammatory and analgesic drugs. Nevertheless, the selection of a correct model requires more detailed information on which animal model can be used to predict a specific clinical pain condition.

Oral Presentations and Poster Session

Painless Nerve Growth Factor: Preclinical Studies

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The clinical application of nerve growth factor (NGF) to prevent or slow human neurodegenerative diseases, such as Alzheimer disease, is limited by the adverse effects of NGF in activating nociceptive responses. We developed a human NGF double mutant (hNGFP61S/R100E), inspired by the HSAN V mutation in the NGFB gene, that has identical neurotrophic properties to human NGF, is traceable against endogenous NGF, and has a greatly reduced ability to activate nociception. The objectives of this study are to assess the therapeutic efficacy of hNGFP61S/R100E in transgenic mice harboring 5 familiar AD-related mutations (5×FAD mice), and to investigate the mechanisms underlying the rescue of neurodegeneration in these mice, as well as the absence or presence of nociception after intranasal delivery. We demonstrate that hNGFP61S/R100E, delivered after the onset of neurodegeneration, induces a complete rescue of spatial memory and synaptic plasticity deficits and a decrease in the plaque load in 5×FAD mice. The mechanisms underlying these effects are linked to a clear reduction pathologic APP processing and astrocytosis and an increase of microglia phagocytic activity. Moreover, we demonstrate that both acute and chronic intranasal administration of painless NGF does not trigger pain in 5×FAD mice, measured at the behavioral level. Thus, these findings confirm that hNGFP61S/R100E is a viable option to increase NGF activity in the brain in a noninvasive way, increasing its pharmacological therapeutic window and provide further proof that the neuroprotective activity of NGF goes well beyond the expected neurotrophic activity on cholinergic neurons.

Maternal Care Alteration in the BTBR T+tf/J Mouse, a Murine Model of Autism

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It has been recently demonstrated that different parental styles can permanently affect DNA methylation, gene expression and neural functions in the offspring, influencing long-term behavior and development of various diseases through epigenetic mechanisms. On such a basis we performed a thorough characterization of the maternal care repertoire of inbred BTBR mice, a validated model for the study of autistic spectrum disorders. Namely, we analyzed BTBR mice during the first 2 wk postpartum as for nest building activity, maternal behavior, and maternal aggression followed by retrieving test, using C57BL/6 mice as controls. Our findings demonstrate significant alterations in maternal care of BTBR mice. In fact, BTBR dams exhibited more pups sniffing and nest building, and lower levels of digging in comparison to controls on postnatal day (PND) 2. As for maternal aggression recorded on PND 7, BTBR dams showed elevated levels of repetitive digging and grooming associated with low levels of nest defense and high levels of passive sniffing. Finally, while in control females time spent in the nest significantly decreased from birth to the second postnatal week, an opposite profile was observed in BTBR females. Our findings supply evidence on the role of early experiences on subsequent development of autistic traits of BTBR mice. Moreover, our study supports the inclusion of standardized protocols for analysis of maternal behavior in the phenotypic characterization of transgenic mouse strains and appears relevant for the correct assessment of gene/environment interactions in neurodevelopmental disorders.

Feasibility of Epidural Catheter Placement in Neonatal Piglets: Description of the Technique

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The aim of this study was to develop and validate the insertion technique of epidural catheters in neonatal piglets. The study was divided in 2 phases: Phase I (anatomic study on 4 cadavers) and Phase II (in vivo application on 6 anaesthetized neonatal piglets). In cadavers, after manual palpation of the transversal processes of the lumbar vertebrae L3 and L4, a 24-gauge Tuohy needle was introduced between the spinous processes with ventrocranial orientation; the catheter was then introduced through the needle within the epidural space for at most 20 cm and visualized under C-arm X-ray. The same operation was performed on anesthetized patients in sternal recumbency; contrast medium was injected to confirm the right placement of the device with X-ray evaluation. In these patients, clinical and anesthesiological parameters were monitored in order to detect any alteration caused by the procedure. The technique allowed us to place the epidural catheter in every piglet. In neonatal subjects, the soft consistency of the bones may be an obstacle to the palpation of the vertebrae. In the study, the operator found the identification of the puncture space harder than in adult pigs, but still possible. The space between L3 and L4 represents a safe window for the insertion of the needle; no clinical signs of hypertension were reported, as suggested by the anesthetic monitoring. The described procedure provides a safe and reproducible route to place epidural catheters in piglets.

Pain Control: Ethical and Legal

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New scientific knowledge is now available about factors influencing animal welfare and the capacity of animals to sense and express pain, suffering, distress, and lasting harm. Therefore, EU Legislation states that is necessary to improve animal welfare in scientific procedures listing a few procedures in relation to their severity degree. However, this list of procedures is insufficient. Now it is necessary that the relief of pain in laboratory animals becomes real. In Italy, a lot of surgical procedures are performed with old drugs lacking high analgesic effects, such as chloral hydrate or thiopental sodium, because researchers do not ask veterinarians for advice. In the presence of surgical wounds it would be advisable to apply a local anesthetic to prevent chewing; the use of analgesics is also helpful to speed recovery after surgery and to promote the mobility and food intake. Also some cases of dermatitis need the use of drugs that reduce itching and the possibility of formation of ulcers due to scratching. Tumors, however, are very different, but in some cases, experimental results may not be affected by the use of analgesics that reduce animal suffering. It is important that all procedures, especially those that may cause pain, suffering, distress, or lasting harm be refined according to the latest scientific knowledge, and that, whenever possible, analgesics be used.

Identification and Treatment of Some Conditions of Discomforts in Laboratory Mice

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The role of the designated veterinarian (DV), according to the new EU legislation, is to supervise the welfare and treatment of animals. The DV is responsible for the prevention, diagnosis, and

treatment of all conditions related to the animal's health and welfare. Legislation also requires that the necessary veterinary care be available at any time to ensure the continuous monitoring of animal welfare. In Italy, the DV may not always be present, and for this reason it is necessary that caretakers be trained to take prompt action. Their duty is to observe the animals daily and perform treatments prescribed by the DV. A well-trained caretaker could identify and treat some well-defined conditions without the DV's previous authorization. The above conditions are only those that commonly could occur in the rodent population housed in the facility: 1) not extensive wounds, 2) anal and vaginal prolapsed, 3) phimosis and paraphimosis, and 4) localized abscesses. The immediate treatments are: 1) chlorhexidine or/and clostebol acetate+neomycin sulfate; 2) euthanasia; 3) depending on the severity, euthanasia or antibiotics; and 4) drain and/or antibiotics. This attitude ensures a quick intervention when the treatment is well-defined. Caretakers should acquire the knowledge to distinguish in animals the abnormal from normal state and the ability to act with appropriate treatment while waiting for the DV's intervention.

Analgesic Treatment in a MCAO Mouse Model

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In imaging studies involving surgical murine models, analgesics should provide postoperative pain relief without compromising experimental objectives. The middle cerebral artery occlusion (MCAO) model is widely used to study the outcomes of cerebral stroke by PET/MRI imaging. Surgical stress and the corticosteroid response are unwanted confounding variables, increasing the between-animal variation. Moreover, it is mandatory to alleviate pain in mice subjected to invasive procedures. Opiates, like buprenorphine, are commonly used for perioperative pain relief in laboratory rodents, and, in contrast to NSAIDs, like meloxicam, seems to have no effects on the infarct volume. Tramadol is a well-studied analgesic in mice that stimulates the μ-opioid receptor, inhibiting serotonin and noradrenalin reuptake. In contrast to other opiates, tramadol does not suppress immune functions and is not a controlled substance. Our pilot study evaluated the utility of tramadol on postoperative recovery in a MCAO mouse model studied by PET/CT imaging. Seventeen C57Bl/6J adult male mice underwent surgical MCAO: 10 mice received only nutritional support and 7 mice also received 500 μL NaCl 0.9% + tramadol (20 mg/kg SID IP) for 48 h. Animal behavior, posture, food and drink intake were monitored. Forty-eight hours after surgery drink and food intake and motor activity increased and antalgic posture was reduced. Kaplan–Meier Curve analysis was assessed in the 7-d postsurgery temporal window. In the analgesic-treated group, survival rate increased by 10% at days 5 and 7 postsurgery. In conclusion, analgesic strategy would not interfere with the desired progress of experimental model, but would improve the postoperative recovery and welfare of mice.

Analgesic Treatment in High-Resolution Ultrasound (HRU)-Guided In Utero Microinjection in Laboratory Mouse

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The mouse is an important model for exploring the developmental consequences of altering gene expression using viral vectors and to validate gene therapies. High-Resolution Ultrasound (HRU) allows guided in utero microinjection of cells, viruses, or other agents in mouse embryos. This procedure requires a laparotomy. Anesthetics and analgesics commonly employed in vivo gene transfer to embryos could affect results of these experiments, the development of embryos at discrete times during pregnancy, litter size, and postnatal health. Information regarding the safety of anesthesia and analgesia during surgery in mouse pregnancy are limited and confounded by many factors. Moreover, buprenorphine or meloxicam could be long-lasting on fetal growth restriction. Instead, the systemic absorption of local anesthetics, including lidocaine, is often limited, so no significant effects on embryos would be expected. We performed HRU-guided microinjection in 8 C57Bl/6J isoflurane-anesthetized pregnant mice at embryonic day 9.5. Postoperative pain relief was attained by intradermal injection of lidocaine (4 mg/kg) along surgical incision for 48 h, in association with "tender loving care" (soft food and bedding, warm environment) as supportive therapy. In all mice, drink and food intake and locomotor activity were restored 30 min after surgery and no antalgic posture was seen. At the end of pregnancy, an average of 8 healthy mice were born. In conclusion, precise design of the anesthetic and analgesic regimens, in association with adequate surgical expertise and animal care, should add significant refinement to the overall quality of experimental outcomes in experimental protocols involving investigations of fetal and neonatal development.

Daily Observations to Control Animal Pain in Laboratory Mice and Rats

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Avoiding animal pain is essential to promote good animal welfare. European legislation on animal use for research considers continuous animal care essential for good health and animal welfare. It is important that all staff recognize animal pain and quickly report to the designated veterinarian (DV). The caretakers have the key role of observing animals daily. They have to be able to distinguish the first signs of pain in animals from their normal state and to inform the DV promptly. Through observation, they are able to gather precise information regarding the

presence of blood/exudates on the walls of the cage, of strange stools, or evident stereotypy. Nevertheless direct observation of animals provides much more information. The external appearance is the first aspect to be taken into account, associated with the body condition score and the observation of the spine and the coat. Mucosa of body orifices (mouth, nose, ear, vagina, prepuce, anus) also needs to be observed. Data about any lesion found on the body have to be reported as follows: correct anatomic position (axillary, abdominally, proximal, cranial), size (to be compared to a universally known object), texture (hard, compact, soft), color, smell, and other adjunctive observations like the presence of blood or pus. It is important that these evaluations be carried out by individuals with a detailed knowledge of the normal and abnormal behavior and appearance of the concerned species. To this end, EU legislation also states that caretakers have to attend various courses on the care and use of animals in research.

Round Table Discussion

Pain and Research

Chair: E D'Amore, Istituto Superiore di Sanità, Rome, Italy

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Animals are still necessary in biomedical research, and therefore researchers have to implement all the measures to guarantee the welfare of laboratory animals and to reduce their pain and distress. Thus, the goal of the new European Directive 63/2010, which was adopted 24 y after Directive 609/86, is to harmonize the legislation of all European Member States, as well as to improve the welfare of animals used in scientific procedures by raising the minimum standards for their housing and treatment in line with the latest scientific developments. The Directive has also officially recognized the principle of the 3Rs, which has been known and applied by the scientific community for many years. Adequate and precise application of the 3Rs principles in biomedical research can reduce both the number of the animals used and the level of pain they experience during procedures. The European Directive 63/2010 supplies a very precise definition of procedure and classifies them according to severity degree in Annex VIII. Another important point, which is not sufficiently specified in the Italian legislation, is the education and professional competence of the personnel performing or supervising procedures. In fact, in Italy there is neither a clear definition of different personnel involved in animal research, such as veterinary surgeons, animal technicians, and animal caretakers, nor a continuing professional training plan. The training of veterinary surgeons in charge of checking animal welfare also plays a predominant role in the new directive. According to the Directive, they should be able to recognize and alleviate pain in laboratory animal and to decide, once the experiment is over, whether animals should be reused or euthanized.

Speakers:
A Affuso, Stazione Zoologica "Anton Dohrn", Naples, Italy; Biogem, Ariano Irpino (AV), Italy
C Bernardi, Accelera, Nerviano (MI), Italy
A Buonacucina, University of Bologna "Alma Mater Studiorum", Italy
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