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The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published till 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is now published under the auspices of the University of Pavia, Italy.
The *European Journal of Histochemistry* is the official organ of the Italian Society of Histochemistry and a member of the journal subcommittee of the International Federation of Societies for Histochemistry and Cytochemistry (IFSHC), and has been an influential cytology journal for over 60 years, publishing research articles on functional cytology and histology in animals and plants.

The Journal publishes Original Papers, Technical Reports, Reviews, Brief Reports, Letters to the Editor, Views and Comments, and Book Reviews concerning investigations by histochemical and immunohistochemical methods, and performed with the aid of light, super-resolution and electron microscopy, cytometry and imaging techniques; attention is also given to articles on newly developed or originally applied histochemical and microscopical techniques.

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- biology of cell development and senescence;
- nerve and muscle cell biology;
- cellular basis of diseases.

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MAIN LECTURES

EARLY LIFE STAGES: ROBUST TOOLS FOR ECOTOXICOLOGICAL INVESTIGATIONS

M. Banni

Laboratory of Agrobiodiversity and Ecotoxicology LR02AGR2, ISA, University of Sousse and ISBM, University of Monastir, Tunisia
E-mail: m_banni@yahoo.fr

Embryonic stages are being increasingly used in ecotoxicology as a sensitive biological model to assess the effects of environmental contaminants. They offer interesting advantages that complement classic models for the evaluation of normal development as well as for the genotoxic effects of exposure to toxicants. Moreover, early life stages are the most susceptible to toxicants and toxicity data using the most vulnerable life stage would offer protection to all life stages in the natural environment. Climate change is one of the greatest environmental challenges and is expected to worsen over the next decades. The mean global sea surface temperature is predicted to rise by 4°C by the end of this century leading to potentially deleterious effects in seawater quality and consequently to marine organisms, due to the tied relation between their metabolic processes and temperature degree. Microplastics (MP) are an emergent pollutant, having become a major concern in recent years due to their physical and chemical properties. Manmade plastic particles constitute the primary MP, and they are used in cosmetics production and in industrial products and thus consitute the main source of pollution by this kind of hazardous. In this lecture, special focus will be done on the effects of climate change and environemental pollutants on the key developmental phases of selected aquatic organisms.

TOXICITY OF MERCURY IN FISH - ARE CELLS OF THE NERVOUS SYSTEM A PREFERENTIAL TARGET?

M. Pacheco

Centre for Environmental and Marine Studies (CESAM) and Department of Biology, University of Aveiro, Portugal
E-mail: mpacheco@ua.pt

The occurrence of mercurial compounds in the aquatic systems is an ostentatious environmental issue, with a historical trail but whose relevance at a global scale is not expected to decrease in the coming decades. Thus, a myriad of fish species is threatened by both organic and inorganic mercury (Hg) forms. The tendency to reason about other organisms by analogy to humans triggered the perception of a risk of neurotoxicity posed by Hg to fish, which was corroborated by research carried out on neuronal and sensory toxicity. The functional importance of the nervous system drew focus on its cells, likely favouring exaggerated interpretations on their vulnerability to Hg. The vulnerability of a specific cell type depends on two sorts of factors: (i) intrinsic features such as those related to cell structure, morphology, physiology and mitotic rate; (ii) extrinsic variables (with respect to the cells in equation) as determinants of Hg toxicokinetics, including the relation with the external milieu and surfaces of uptake, blood perfusion of the tissue/organ, composition of the extracellular matrix and the presence of blood-organ protection barriers. Both organic and inorganic Hg forms showed to be able to cross fish blood-brain-barrier (BBB), affecting the brain cells. However, BBB and blood–retinal-barrier (BRB) limited the influx of inorganic Hg, respectively to the brain and to the eye wall, when compared, for instance, to the liver, highlighting their shielding role and pointing out a lower susceptibility of neuro-sensory structures. Most *in vivo* fish studies carried out in this context approached the tissue- or organ-specific susceptibility towards Hg accumulation and toxicity, devaluing, at least implicitly, that most tissues/organs integrate different types of cells, often with peculiar intrinsic characteristics. Though cells do not exist in isolation, these approaches hamper the full elucidation of cell-specific vulnerabilities. As an exception to this research profile, a recent investigation shed some light on this question, comparing two cell types sharing the same cellular microenvironment, viz. neurons and glial cells, revealing that both organic and inorganic Hg forms targeted preferentially the formers. A critical overview of the laboratory studies that can contribute to answering the question/challenge posed ad initium will be presented, but an unequivocal answer cannot be promised.

ABSTRACTS

THE ROLE OF EPIGENETIC MECHANISMS IN NANOTOXICITY

F. Gamberoni[1], M. Borgese[2], S. Serio[4], R. Papait[1,3], R. Gornati[1], G. Bernardini[1]

[1]*Department of Biotechnology and Life Science, University of Insubria, Varese, Italy;* [1]*Department of Medicine and Surgery, University of Insubria, Varese, Italy;* [3]*IRCCS Humanitas Research Hospital, Milan, Italy*
E-mail: f.gamberoni@uninsubria.it

In the last years the use of nanoparticles (NPs) has spread over different fields (*e.g.*, industry, environment, electronics, cosmetics, and biomedicine),[1] and thus, this makes the assessment of their toxicological risk an important aspect to guarantee safety to the environment, consumers, and workers. DNA damage, generation of reactive oxygen species, mitochondrial dysfunction, apoptosis, and inflammation are the most common toxic effects of NPs. Lately, many progresses have been made in the field of nanotoxicity, but despite that, a question that remains unanswered is the role of epigenetic mechanisms in nanotoxicity. Epigenetics are a set of mechanisms that define the status of gene expression without changing the DNA sequence. Thanks to the use of 'omics' approaches to study the epigenome (*e.g.*, chromatin immunoprecipitation coupled with massively parallel sequencing − ChIP-seq) and the transcriptome (*e.g.*, RNA-sequencing − RNA-seq), it is clear that the epigenetic marks determine the transcription programs underlying cell differentiation and the maintenance of tissue homeostasis. Moreover, the cause of many human diseases can be found in the alteration of the genomic distribution of epigenetic marks, which affects the transcriptome. Therefore, in this regard, we have analyzed the genomic distribution of four histone modifications (H3K27ac, H3K4me1, H3K9me2, and H3K27me3) and the transcriptome in NIH- 3T3 cells exposed to two iron-based magnetic NPs, CSIC-9 and ICMA-4, synthesized by the European HOTZYMES consortium. We found that the variation in the genomic distribution of H3K27ac directly caused a change in the transcription pattern. This is primarily due to the modulation of the activity of enhancers: there are specific epigenetic signatures underlying the transcriptional responses to each NP that are not due to iron release from NPs. These results suggest that an alteration of the epigenetic landscape is a key mechanism in defining the gene expression program changes resulting in nanotoxicity.

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EFFECTS OF NATURAL NANOPOWDERS ON ZEBRAFISH EMBRYOS AFTER SHORT-TERM TEST: PRELIMINARY RESULTS

R. Pecoraro[1], E.M. Scalisi[1], G. Giuffrida[1], S. Indelicato[1], M. Contino[1], F. Capparucci[2], G. Lanzafame[1], P. Mazzoleni[1], G. Barone[1], M.V. Brundo[1]

[1]*Department of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy;* [2]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy*
E-mail: roberta.pecoraro@unict.it

Volcanic ashes have an important falls because of the big distances they can achieve, sometimes covering wide areas.[1] Many studies regarding effects on living organism have been done in the last decades. The aim of this work is evaluating the potential toxic effects of volcanic natural nanoparticles deriving from two different lava flows of Mount Etna on *Danio rerio* (zebrafish) development. Zebrafish is a freshwater fish considered one of the best animal model to obtain useful response also for human. An acute toxicity test was carried out following international guidelines.[2] Increasing concentrations of nanopowders (64 nm in dimension) characterized by X-Ray Fluorescence Spectrometry at University of Ottawa (Canada), were tested on eggs coming from the Sicilian Centre for Experimental Fish Pathology (at Department of Veterinary Sciences, University of Messina). During the exposure period (96 h), four endpoints (embryo coagulation, lack of somites, non-detachment of the tail and lack of heartbeat) were analyzed every 24 h. The response to biomarkers of exposure as Metallothioneins (MT) and Heat Shock Protein 70 (HSP70) was evaluated. Data showed that the exposure of zebrafish embryos to volcanic ashes doesn't affect embryonic development even at the highest concentrations since the embryos were viable and physiologically hatched at 72 h. A slight positivity to MT was found only in the anterior part (head) of the exposed larvae compared to controls. No positivity was found for HSP70. These data could suggest that in zebrafish, a short-term exposure to volcanic ash is not toxic, at least at the tested concentrations, so further studies are needed to confirm these preliminary results and also to give more details about a chronic exposure since there is scant of literature on it.

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COMBINED EFFECTS OF MICROPLASTICS AND CADMIUM ON RAT TESTICULAR ACTIVITY

M. Venditti[1], M. Ben Hadj Hassine[2], I. Messaoudi[2], S. Minucci[1]

[1]*Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy;* [2]*Higher Institute of Biotechnology, University of Monastir, Monastir, Tunisia*
E-mail: massimo.venditti@unicampania.it

Among the plethora of harmful substances affecting human and animal health, microplastics (MP) are attracting increasing attention, as all organisms are continuously exposed to them[1]. MP affect mammalian testicular physiology by inducing inflammation, oxidative stress, impairment of the seminiferous epithelium (SE) cytoarchitecture, and blood-testis barrier (BTB) integrity, ultimately, leading to abnormal differentiation of mature gametes.[2-4] Another aspect to be considered is that MP inevitably coexist with other chemicals and, due to their high surface area/volume ratio and hydrophobicity, can adsorb and transport them, thanks the "Trojan horse" effect.[5] This study expands the knowledge on the combined impact of MP and cadmium (Cd), at cellular and molecular level, on the progression of the differentiative events of germ cells (GC) into mature gametes. Oral treatment with both substances caused testicular impairment, evidenced by histological and biomolecular alterations, such as MP accumulation in the SE, imbalance of oxidative status, and reduced sperm quality.

Importantly, the BTB integrity was compromised, as revealed by the down-regulation of levels of structural proteins (occludin, VANGL2, and Cx43) and activation of regulative kinases (Src and FAK). Interestingly, MP activate the autophagy pathway in GC, to reduce damaged organelles and molecules, in an attempt to avoid apoptosis. Surprisingly, the results obtained with the simultaneous Cd+MP treatment showed more harmful effects than those produced by MP alone but were less severe than with Cd alone. This might be due to the different ways of administration to rats (oral gavage for MP and in drinking water for Cd), which might favor the adsorption, in the gastrointestinal tract, of Cd by MP, which, by exploiting the Trojan horse effect, reduces the bioavailability of Cd.

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EVALUATION OF THE EXPOSURE TO MICROPLASTICS AND ASSOCIATED CONTAMINANTS IN *IN VITRO* AND *IN VIVO* MODELS

A. Saraceni[1,2], E. Cottone[1], B. Mognetti[1], V. Schiavo[2], S. Palermo[2], F. Geddo[1], G. Querio[1], S. Antoniotti[1], M.P. Gallo[1], G. Merlo[2], F. Dal Bello[2], P. Bovolin[1]

[1]Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy; [2]Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy
E-mail: astrid.saraceni@unito.it

Plastic pollution is an emerging global concern. In addition to damages caused by large plastic wastes, there is increasing attention on Microplastics (MPs), ubiquitous environmental pollutants potentially dangerous for human and wildlife health. Moreover, there is growing evidence of the potential of MPs to become vectors of a wide range of contaminants, such as Endocrine Disrupting Chemicals (EDCs), possibly responsible for some indirect effects. In our study, we focused on 5 μm and 0.5 μm polystyrene microplastics (PS-MPs), both pristine and functionalized (-COOH), as carboxylated MPs may mimic environmentally aged MPs. We assessed the impact of MPs exposure both *in vitro*, using human liver cancer, murine pre-adipocyte, and bovine aortic endothelium cell lines, and *in vivo*, in zebrafish (zf) larvae. MPs were internalized by cultured cells and ingested by zf larvae, accumulating in the intestine. *In vitro* cytotoxic effects were observed only in cells exposed to very high concentrations of functionalized MPs. In zf larvae survival, hatching, and heartbeat were monitored until 6 dpf; virgin-MPs exposure did not lead to macroscopic deleterious effects on developing organisms. To investigate the potential role of MPs as vectors of contaminant chemicals, the adsorption of Bisphenol A (BPA), a well-known EDC, on PS-MPs was tested using a UHPLC-tandem MS method. Following a 24 h incubation, the adsorption yield of BPA (25 μM) on PS-MPs was about 50%. In our experimental models, the exposure to MPs preadsorbed with non-toxic concentrations of BPA had no toxic effects. We are currently testing other biological effects, including the induction of oxidative stress, changes in triglyceride accumulation, and alterations in gene expression. Our results suggest that PS-MPs are ingested by zf larvae and internalized in cultured cells, leading to toxic effects only at high concentrations. More in-depth investigations are needed to evaluate subtle metabolic and developmental effects of contaminant-associated MPs.

EFFECTS OF VANADIUM DURING DEVELOPMENT OF SEA URCHIN EMBRYOS IN A CLIMATE CHANGE PROSPECTIVE

R. Chiarelli[1], F. Geraci[1], R. Scudiero[2], M.C. Roccheri[1], C. Martino[1]

[1]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy; [2]Department of Biology, University Federico II, Naples, Italy
E-mail: roberto.chiarelli@unipa.it

The increasing industrial use of vanadium (V), as well as its possible pharmaceutical use in various diseases, has intensified its environmental release, making it an emerging pollutant. Biological effects of metal pollution can be modified by higher seawater temperatures[1,2]. *Paracentrotus lividus* embryos exposed to V show altered phenotypes, skeletal absence or malformations, cell stress response mediated by HSPs, autophagy and apoptosis, and modulation of metal related proteolytic activities.[3,4,5] Here, we have extended our V-toxicity studies to determine embryo response to two different temperature increases: +3° (21°C) and +6° (24°C) with respect to the physiological developmental temperature (18°C). These temperatures agree with near-future (2100) temperature projections for ocean warming and current marine heatwaves events for the Mediterranean Sea (IPCC, 2019). At 24 h and 48 h development accelerates in a temperature-dependent manner. V-exposed embryos showed alternative developmental phenotypes. Rising temperature caused an increased V intake in embryo cells and a reduction in intracellular calcium. Zymography assays showed that embryos modulated proteolytic activities and the levels of several metalloproteinases. The cytoprotection triggered by temperature increase was tested by HSP60 and HSP70 levels. HSPs acted primarily during early development (8 h) and it was mainly mediated by HSP60. The activation of cell-selective apoptosis resulted as a last defense strategy, probably to in part defend the development program. Our results indicate that sea urchin embryos can activate different defense strategies to overcome the negative effects of V exposure on embryo development. The effects of global warming could be met up to a thermotolerant threshold. However, the negative synergistic effects may become irreversible.

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CYTOTOXIC ACTION INDUCED BY SINGLE AND COMBINED EXPOSURE OF REALISTIC DOSES OF CAFFEINE AND SALICYLIC ACID IN MUSSEL *MYTILUS GALLOPROVINCIALIS*

M. Galati[1], G. De Marco[1], S. Afsa[2], B. Billè[1], A. Giannetto[1], T. Cappello[1], M. Maisano[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy; [2]Research Unit of Analysis and Process Applied to The Environment – APAE (UR17ES32), Higher Inst. of Applied Sciences and Technology of Mahdia, University of Monastir, Monastir, Tunisia
E-mail: mariachiara.galati@studenti.unime.it

The consumption of active pharmaceutical products (PhACs) has increased globally.[1] Most of the PhACs are not fully metabolized, and so are excreted in an active form. Due to the inadequacy of wastewater treatment plants (WWTPs) towards all types of PhACs,[2] these compounds easily reach the aquatic environment, becoming potential micropollutants for biota. Among PhACs, caffeine (CAF), a nervous stimulant, and salicylic acid (SA), a non-steroidal anti-inflammatory drug (NSAID), are the most abundant.[3] Therefore, their single and combined (SA+CAF) effects were evaluated at different time-points on the filter-feeder mussel *Mytilus galloprovincialis* following a short-term exposure at their environmentally relevant concentrations (ng/L-µg/L) for 12 days, selecting the digestive gland as target organ. Absence of histomorphological damage parallelly with haemocyte infiltration highlighted an inflammatory response and activation of systemic defensive mechanisms in response to the tested PhACs. This was also supported by induction of the oxidative stress pathway, as revealed by the modulated activity and gene expression of a battery of oxidative stress end-points (SOD, CAT, GST). It is interesting to note that, under realistic conditions, the two compounds alone provoked the most pronounced effects whereas, when combined, the stimulating action of CAF was compensated by the inhibitory action of SA. Overall, these results enlarge knowledge on pharmaceuticals effects on non-target organisms and emphasize the need for proper environmental risk assessment finalized to enhance the efficacy of WWTPs towards PhACs.

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CELLULAR MECHANISMS ALTERED BY ENVIRONMENTAL DOSAGES OF ANTI-INFLAMMATORY AND ANTIVIRAL DRUGS IN MARINE MUSSELS

G. De Marco, M. Galati, B. Billè, S. Abouda, K. Riolo, V. Parrino, T. Cappello, M. Maisano

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
E-mail: giuseppe.demarco@unime.it

In recent years, pharmacologically active compounds (PhACs) have accounted among emerging classes of contaminants. Hence, due to their wide use and the general inefficiency of wastewater treatment plants (WWTPs) against this group of pollutants, PhACs are usually detected in the environment at concentrations in the ng/L-µg/L range, and therefore referred as 'micropollutants'. Previous studies have focused on the effects of PhACs on non-target organisms.[1,2] However, the Covid-19 pandemic has certainly increased the release of PhACs into the environment, especially anti-inflammatory and antiviral drugs. Therefore, this study evaluated the effects of realistic concentrations[3,4] of the anti-inflammatory dexamethasone (C0: 0, C1: 4 ng/L, C2; 40 ng/L; C3: 400 ng/L, C4: 2000 ng/L), and of the main metabolite of the antiviral Remdesivir, namely GS-1224 (C0: 0, C1: 4 ng/L, C2: 40 ng/L; C3: 400 ng/L, C4: 2000 ng/L) on marine mussel *Mytilus galloprovincialis* at different exposure times (T0: 0 days, T3: 3 days, T6: 6 days, T12: 12 days). By assessment of endpoints related to the oxidative stress (CAT, GST, SOD, LPO) and neurotoxicity (AChE), a pro-oxidative effect was observed at the different time-points for both PhACs tested, with an inhibition of AChE activity prompted by the higher concentrations of dexamethasone. These results highlight the necessity to mitigate the environmental occurrence of these two PhACs even at low concentrations, as well as to carry out further investigations to better elucidate the possible onset of cellular pathways impairment in non-target organisms. Overall, the final purpose of these studies is to provide useful input for improving the efficacy of WWTPs, promoting the use of novel materials able to trap these emerging contaminants.

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MORPHO-FUNCTIONAL IMPAIRMENT OF INTESTINE AND SKELETAL MUSCLE IN *XENOPUS LAEVIS* EMBRYOS EXPOSED TO DELORAZEPAM

C. Fogliano[1], R. Carotenuto[1], G. Rusciano[2], A. Sasso[2], C.M. Motta[1], C. Agnisola[1], B. Avallone[1]

[1]Department of Biology, University of Naples Federico II, Naples, Italy; [2]Department of Physics, University of Naples Federico II, Naples, Italy
E-mail: chiara.fogliano@unina.it

Psychotropic drugs, such as benzodiazepines, are among the most frequently found pharmaceutical residues in aquatic matrices.[1,2] An increasing number of studies are reporting their harmful effects on the behavior and physiology of non-target adult species, but little information is available regarding the impact on developing organisms, exposed since early stages. Improper activation of GABA and TSPO receptors during embryonic development is, in fact, likely to induce relevant consequences on the morphogenesis and behaviour.[3] Also altering mitochondrial function thus reducing the energy available and improperly activating apoptotic/proliferative pathways.[4] Our previous investigation highlighted, in *Xenopus laevis* embryos, increased mortality and developmental abnormalities in the retina, intestine, and tail, supporting an interference mechanism with early developmental genes.[5] Based on this evidence, we extended the investigation on the intestine and tail skeletal muscle through microscopic and ultrastructural investigations. Data were integrated with Raman spectroscopy analysis and conventional behavioural tests, to evaluate how much damage influences the interplay and/or functionality of muscles, sensory organs and the nervous system. Results confirm previous evidence of the negative impact of delorazepam on early development and

return an alarming picture of the amphibians and other aquatic species' survival potentialities in a benzodiazepine-contaminated environment.

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GLYPHOSATE IMPAIRS STRUCTURAL AND FUNCTIONAL ORGANIZATION OF OVARIAN FOLLICLES IN THE LIZARD *PODARCIS SICULUS*

L. Rosati, T. Chianese, V. De Gregorio, R. Cominale, R. Leandri, CM. Motta, R. Scudiero

Department of Biology, University Federico II, Naples, Italy
E-mail: luigi.rosati@unina.it

Glyphosate (Gly) is the world's leading broad-spectrum, non-selective herbicide for weed control. Gly blocks the biosynthesis of aromatic amino acids in plants: as this pathway is absent in animals, Gly was considered safe for animals. Over the years, however, evidence has accumulated on the toxic effect of Gly in animals as well. Our recent studies aimed to evaluate the Gly effects on the lizard *Podarcis siculus*. This lizard is a good bioindicator in ecotoxicology, because it can be contaminated in its natural habitat by pesticides, which modify its health and give rise to measurable biological responses.[1] We previously proved that Gly exposure affected the morpho-physiology of both liver and testis of *P. siculus*. In the liver, Gly induced severe fibrosis, activation of the antioxidant defence machinery and xenoestrogenic effects;[2] in the testis, Gly altered testicular morphology, affected spermatogenesis, and changed the localization of estrogen receptors.[3] In the research presented here, we focused our attention on the toxic effect of Gly on the lizard ovary. To this end, we studied the structural and functional organization of ovarian follicles after oral exposure to 0.05 and 0.5 μg/kg of Gly for 3 weeks. By cytological analyses, we determined the effects on the number and conditions of germ cells and follicles; by immunocytochemistry we detected alterations in PCNA expression, indicative of alterations in proliferation rate; in E-cadherin localization, indicative of a loss of contact between follicle cells; in estradiol a and b receptors, indicative of an interference with the endocrine axis. The results demonstrate that Gly, at both doses tested, profoundly interferes with ovarian function. It induces germ cells recruitment and alters follicular anatomy by anticipating pyriform cells apoptotic regression. It also induces thecal fibrosis and affects oocyte cytoplasm and zona pellucida organizations. Functionally, Gly stimulates the synthesis of estrogen receptors, suggesting a serious endocrine disrupting effect.

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DETRIMENTAL EFFECTS OF CADMIUM ARE COUNTERACTED/PREVENTED BY D-ASPARTATE IN RAT TESTIS

A. Santillo[1], M. Venditti[2], D. Latino[1], M.M. Di Fiore[1], S. Minucci[2], I. Messaoudi[3], S. Falvo[1], G. Chieffi Baccari[1]

[1]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Caserta, Italy; [2]Department of Experimental Medicine, Section Human Physiology and Integrated Biological Functions, University of Campania "L. Vanvitelli", Naples, Italy; [3]LR11ES41: Genetics, Biodiversity and Valorization of Bioresources, Higher Institute of Biotechnology, University of Monastir, Monastir, Tunisia
E-mail: alessandra.santillo@unicampania.it

Cadmium (Cd) is known to cause severe testicular injury, documented by histological and biomolecular alterations, such as decreased serum testosterone (T) level and impairment of spermatogenesis.[1] Several studies aimed to identify compounds that can counteract or prevent its harmful effects for use in new therapeutic approaches. In this context, we evaluated the possible counteractive/preventive action of D-Aspartate (D-Asp), known for its ability to induce T biosynthesis and spermatogenesis progression,[2] Our results confirmed that Cd affects testicular activity, as documented by the reduction of serum T concentration and the protein levels of steroidogenesis (StAR, 3β-HSD, and 17β-HSD) and spermatogenesis (PCNA, p-H3, and p-SYCP3) markers. In addition, higher protein levels of cytochrome C and caspase 3, along with the number of cells positive to TUNEL assay, indicated the intensification of the apoptotic process. D-Asp, administered simultaneously to Cd or for 15 days before the Cd-treatment, reduced the oxidative stress induced by the metal, alleviating the consequent harmful effects. Interestingly, the preventive action of D-Asp was more effective than its counteractive effect. A possible explanation is that giving D-Asp for 15 days induces its significant uptake in the testes and pituitary gland, reaching the concentrations necessary for optimum function. In summary, this report highlights, for the first time, the beneficial role played by D-Asp in both counteracting/preventing the adverse Cd effects in the rat testis, strongly encouraging further investigations to consider the potential value of D-Asp also in improving human testicular health and male fertility.

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ARE INVERTEBRATE COMPLEMENT REGULATORS HYPERVARIABLE? NEW EVIDENCE FROM NANOPORE TECHNOLOGY

N. Franchi[1], G. Sales[2], A. Vezzi[2], S. Sacchi[1], D. Malagoli[1]

[1]Department of Life Sciences, University of Modena and Reggio Emilia; [2]Department of Biology, University of Padua, Italy
E-mail: nicola.franchi@unimore.it

We know that practically all animals are capable of having an immune reaction against tissues transplanted from different organisms even belonging to the same species. In a few cases we have described specific molecular apparatuses for the recognition of

histocompatibility. The question we ask ourselves is: do all organisms have their own molecular histocompatibility apparatus or is there a molecular apparatus capable of operating, more or less specifically, a recognition of "non-self" tissues? We believe that this universal histocompatibility mechanism may be the complement system and its regulators. To date, we know very little about Complement Regulators and all the information comes from Vertebrate animals. What we do know about this class of molecules is that they are able to block C3 preventing phagocytosis of the cells they are on. Vice versa they favor the elimination of cells in which they are absent or not recognized. This last aspect is what we are trying to investigate: could the non-functioning of these complement regulators be due to their sequence hypervariability which therefore makes them specific for each single organism? Illumina technology, on which most of today's transcriptomes are based, only allows us to guess that this variability may exist, but cannot confirm it. That's why we present the first data obtained with Nanopore third-generation sequencing technology.

CHEMICAL DEPLETION OF PHAGOCYTIC HEMOCYTES TRANSIENTLY HALTS THE REGENERATION OF THE CEPHALIC SENSORY TENTACLE IN ADULT *POMACEA CANALICULATA* AND AFFECTS THE EXPRESSION OF IMMUNE-RELATED MOLECULES IN THE TENTACLE BLASTEMA

G. Bergamini[1], S. Sacchi[2], A. Ferri[3], N. Franchi[2], M. Montanari[2], M. Ahmad[3], M. Nasi[4], M. Cocchi[3], D. Malagoli[2,5]

[1]Department of Biology and Evolution of Marine Organisms, Zoological Station "Anton Dohrn", Naples, Italy; [2]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; [3]Department of Chemical and Geological Sciences, University of Modena and Reggio Emilia, Modena, Italy; [4]Department of Surgical, Medical and Dental Sciences, University of Modena and Reggio Emilia, Modena, Italy; [5]NBFC, National Biodiversity Future Center, Palermo, Italy
E-mail: davide.malagoli@unimore.it

Regenerative capacity is variously represented in the animal kingdom and it is limited in mammals.
In consideration of the numberless applications of a controlled regeneration in humans, the adult regeneration bases have been studied in vertebrates and invertebrates, evidencing the importance that the immune system could play. After amputation, granular hemocytes infiltrate the blastema of regenerating cephalic tentacles of the freshwater snail *Pomacea canaliculata*.[1] Here, the snail phagocytic hemocytes were depleted by injection of clodronate liposomes, and the effects on the tentacle regeneration were investigated *via* histological and qPCR analyses. Flow cytometry analysis demonstrated that clodronate liposomes targeted phagocytic hemocytes, mainly depleting the hemolymph of large hemocytes. In correspondence with the phagocyte depletion, tentacle regeneration was halted, and it started at the expected pace after 24 h, when clodronate liposome effects on the hemolymph were no more visible. Consistently with histological observations, also the expression of the immune related genes *Pc*-Hemocyanin and *Pc*-Transglutaminase, *i.e.*, markers of hemocyte-mediated functions like oxygen transport, antibacterial activity and clotting, was reduced in the tentacle blastema, and recovered to non-injected control snail levels only after 24 h. In all, our data indicate that phagocytic hemocytes can play an active and non-redundant role in promoting the early onset of tentacle regeneration in *P. canaliculata*, and that first phases of tentacle regeneration may represent a useful biological context for studying immune and non-immune functions of specific hemocyte subpopulations.

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FROM THE MOLECULAR TO INDIVIDUAL LEVEL: AN INTEGRATIVE APPROACH TO INVESTIGATE INTO THE MECHANISMS OF NANOPLASTICS TOXICITY DURING *CIONA ROBUSTA* EMBRYOGENESIS

M.C. Eliso[1,2], E. Bergami[3], L. Bonciani[4], R. Riccio[4], G. Belli[4], M. Belli[4], T. Cappello[1], I. Corsi[5], A. Spagnuolo[2]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina; [2]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples; [3]Department of Life Sciences, University of Modena and Reggio Emilia, Modena; [4]BioChemie LAB, Campi Bisenzio, Florence; [5]Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy
E-mail: maeliso@unime.it

The growing concern around nanoplastics (NPs, <1 μm) has led to a wide investigation on their effects on marine life. A significant amount of recent studies has focused the attention on their mechanisms of action for assessing and predicting the toxicological effects at organism and population levels. Here, we applied an integrative approach to develop an Adverse Outcome Pathway (AOP) upon acute exposure to polystyrene nanoparticles (PS NPs) as proxy for nanoplastics, of 50 nm amino-modified particles (PS-NH_2; 10 and 15 μg mL^{-1}), as proxy for NPs, during the embryogenesis of the chordate *Ciona robusta*. Transcriptomic data and *in vivo* experiments were assembled into two putative AOPs, identifying as key events the adhesion of PS-NH2 as (molecular) initiating event, followed by oxidative stress, changes in transcription of genes involved in glutathione metabolism, immune defense, nervous system, transport by aquaporins and energy metabolism, morphological defects, increase in reactive oxygen species level and impaired swimming behavior. As final adverse outcomes, altered larval development, reduced metamorphosis and inhibition of hatching were identified. Moreover, chemical analysis showed the release of byproducts such as styrene monomers and 2,4-di-tert-butylphenol (DTBP) in the exposure media, which could have had a potential role in the observed outcomes. This study provides new insights into the understanding of PS-NH_2 toxicity on early stages of marine life and could potentially be used for environmental risk assessment purposes.

MICROPLASTICS FROM FFP2 MASKS AFFECT *PARACENTROTUS LIVIDUS* DEVELOPMENT

F. Vari[1], D. Vardanyan[1], L. Valli[2], G. Giancane[2], S. Tacconi[3], L. Dini[1]

[1]Department of Biology and Biotechnologies Charles Darwin, Sapienza University of Rome, Rome, Italy; [2]Department of Biological and Environmental Science and Technology, University of Salento, Lecce, Italy; [3]CarMeN Laboratory (UMR INSERM 1060/INRA 1397), Lyon-Sud Faculty of Medicine, University of Lyon, Pierre-Bénite, France
E-mail: francesco.vari@uniroma1.it

Over the last three years, since the outbreak of the SARS-CoV-2 pandemic, there has been a massive production and use of FFP2 masks. The use of masks has reduced the spread of SARS-CoV-2 in society; however, their improper disposal is becoming an emerging source of microplastic pollution.[1,2] As FFP2 masks have a multi-layered structure with the base material being two plastic polymers, PP, polypropylene, and PE, polyethylene, their toxicity to the environment and human health should be tested. Plastic pollution is pervasive and affects all terrestrial ecosystems, from fresh and marine waters to the atmosphere. Of particular concern are nanoplastics in the form of beads and fibres (less than 1000 nm in size) released from environmental degradation of micro/macroplastics or produced directly in nanoscale size. Due to their small size and ability to cross intestinal barriers, nanoparticles are highly bioavailable to organisms, including humans. Therefore, the aim of this work is the toxicological evaluation of improperly disposed FFP2 masks in the marine environment.[3] FFP2-derived nano-/microplastics were first characterized by light microscopy and Raman spectroscopy for their shape, size, and amount and then, evaluated for their toxicity on the sea urchin development of *Paracentrotus lividus*. A further comparison with the effects of different concentrations of commercially available 100 nm polystyrene nanoparticles (PS NPS) was also performed. Many different structural, molecular and biochemical parameters such as gamete quality and fertilisation rate, developmental rate, morphological malformations, embryo death rate, oxidative stress levels, changes in oxygen respiration consumption and gene alterations were studied. Overall, the nano/microplastic produced by both the degradation of the FFP-2 mask and the commercial bead were found to have a negative effect on almost all the parameters studied, depending on the concentration. In fact, the extent and type of damage may have been slightly different, probably due to the abundance of fibres in the FFP2-derived nano-/microplastics. Given the conservation of many *P. lividus* genes in humans, these microplastics could also pose a threat to human health.

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CYTOTOXIC EFFECTS INDUCED BY MICRO AND NANOPLASTICS IN GILLS OF *MYTILUS GALLOPROVINCIALIS*

R. Romano[1,2], L. Rosati[3], F. Ferrigno[1], M. Russo[1], T. Chianese[3], S. Belardo[1], R. Rozza[1], G.F. Russo[1,2], R. Sandulli[1], M.C. Motta[3], P. Simoniello[1,2]

[1]Department of Science and Technologies, University of Naples "Parthenope", Naples; [2]International PhD Programme UNESCO Chair "Environment, Resources and Sustainable Development"; [3]Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: rossana.romano001@studenti.uniparthenope.it

Plastic is one of the main present pollution sources owing to its global distribution with potential ecotoxicological risk. In the aquatic environment, the degradation products of plastic waste are increasing. More and more interest has been paid in the last few decades on their effect on marine environments and on the phenomenon of biomagnification along the marine food chain.[1] In this work, *M. galloprovincialis* was used as a bioindicator model to analyse the cytotoxic effects induced on marine organisms by Microplastics (MPs) and Nanoplastics (NPs). Mussels were exposed to polystyrene (5 or 0.1 μm) for 1, 3, or 11 days. Expositions times and plastics' concentrations were chosen according to realistic environmental states.[2] Gills were processed for light microscopy to analyse how MPs and NPs could interfere with the tissues' organization and function. Histological analysis demonstrated significant alterations in the gills after short and long-term treatments. Disorganization on the septum and lamellar structures were observed, and melanin and lipofuscin infiltrations were detected. PAS staining revealed abnormal distribution and an increasing number of mucus cells, especially after NPs exposure. MPs and NPs affected proliferation: immunocytochemistry investigations by PCNA demonstrated an increasing number of positive cells after treatment. HSP70 detection indicated marked cellular stress response. Polystyrene induces severe alterations in the morphological organization and cellular functionality process as cell cycle and cellular stress. It is unrealistic to think about solving the plastic waste problem in short time, plastic production will continue to cause damage to the aquatic ecosystem. Monitoring its dispersion represents a matter of urgency for environmental protection.[3]

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MICROPLASTICS IDENTIFIED, FOR THE FIRST TIME, IN THE YOLK AND EMBRYOS OF COMMON CUTTLEFISH FROM THE CENTRAL ADRIATIC SEA: EFFECTS ON EMBRYOS AND HATCHLINGS' DEVELOPMENT

G. Chemello, V. Faraoni, V. Notarstefano, O. Carnevali, G. Gioacchini

Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy
E-mail: giorgia.gioacchini@univpm.it

Microplastics (MPs) reaching the marine environment could be accidentally ingested by the aquatic biota entering the food chain with possible negative effects on the health of several species.[1] This aspect represents a potential risk for both wildlife and humans when involves species of high commercial interest such as the common cuttlefish (*Sepia officinalis*). MPs have been already observed in adult specimens of common cuttlefish opening the speculation on MPs' fate once ingested.[2] One of the most plausible hypotheses is the vertical transmission of MPs from the female to the eggs during the oogenesis. The present study investigated, for the first time, the presence of MPs in cuttlefish embryos and yolk and their correlation with embryonic development. Cuttlefish eggs from four different sites along the Marche region were sampled. Embryos and hatchlings' biometric parameters were analyzed and their internal structures and organs were evaluated through histological analysis, while embryos and yolk samples were processed for MPs detection. MPs were observed in both embryos and yolks from all the sampling sites (size < 5 μm). The presence of MPs in yolk samples supported the maternal-transfer hypothesis, while the observation of the same MPs type in yolk and embryos from the same eggs suggested their assimilation by the embryo. MPs presence was not associated with any impairment in both embryos and hatchlings' internal structures. However, the highest number of MPs (in both yolk and embryo samples) were found in the site

where the lower hatchlings' size was observed. Conversely, the highest embryos' mantle length was observed in hatchlings from the site with the lower number of MPs. The possible effects of microplastic presence on the health and development of cuttlefish embryos should be investigated considering their reproductive strategy which leads to the death of adult specimens at the end of a single spawning season.

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COMPARISON OF SALICYLIC ACID AND CAFFEIN EFFECT ON THE DIGESTIVE GLANDS MICROTUBULES OF *MYTILUS GALLOPROVINCIALIS*

M. Dara[1], F. Bertini[1], A. Gambino[1], C. La Corte[1], M. Galati[2], M. Maisano[2], M. Cammarata[1], M.G. Parisi[1]

[1]Department of Earth and Sea Sciences, University of Palermo, Palermo; [2]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
E-mail: federica.bertini02@unipa.it

Pharmaceutically active compounds (PhAC) represent the main class of micropollutants found in aquatic environments. Environmental contamination of PhACs is a consequence of their wide use and failure to remove wastewater treatment plants. Therefore, PhACs pose serious concerns for their harmful effect on non-target organisms. Among the PhACs, caffeine (CAF) and salicylic acid (SA) were found to be constantly present in the aquatic environment at higher concentrations. To date, their impact has been extensively studied, while little information exists on their effects as mixtures. Their biological effects have been evaluated in various aquatic organisms, indeed, PhACs have the potential to trigger different types of biological responses in non-target organisms. Since damage in cellular and tissue organization is frequently observed in organisms after exposure to a variety of stressors, the assessment of changes in histomorphological characteristics has become a fundamental approach in ecotoxicological studies. This study was therefore designed to evaluate the effects of CAF and SA, and their mixture, in the bivalve mollusc *Mytilus galloprovincialis* and to elucidate the time-dependent biological effects induced in mussels by subchronic exposure at environmentally realistic concentrations. The impact was evaluated by observing the microtubule modification of the digestive gland by histological examination. The analyses were conducted by an imaging program (ImageJ) that evaluates the modification of the epithelium and the tubular lumen of the digestive tubules. This study provides insights into the real impact of PhACs on non-target organisms, shedding light on their possible effects occurring in mixtures in the environment. After exposure to various conditions, changes in the microtubule structure of the digestive gland of treated animals were observed relative to the control. The results of this study can therefore be useful for defining effective measures to be adopted in the context of eco-pharmacovigilance programmes.

A MESOCOSM STUDY: BEHAVIOURAL AND PHYSIOLOGICAL STRESS RESPONSES OF *CHERAX QUADRICARINATUS* AFTER EXPOSURE TO ACOUSTIC SIGNAL

C. De Vita[1,2], G. Buscaino[2], M. Mauro[1], M. Arculeo[1], V. Arizza[1], M. Vazzana[1]

[1]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo; [2]Institute of Anthropic Impact and Sustainability in Marine Environment (IAS) - CNR National Research Council, Torretta Granitola (Trapani), Italy
E-mail: clarissa.devita@unipa.it

Anthropogenic sound is recognized as a major environmental stressor that, in the long term, can have negative consequences on species. In recent years, there has been growing attention to the potential negative impact of noise pollution on species, with great concern about the significance of impacts on aquatic animal life. This study examined the effects of acoustic stress on the behavior and haemolymphatic parameters of the freshwater shrimp *Cherax quadricarinatus*. The experiment was conducted in a tank equipped with an audio and video recording system using ten groups (five control and five test) of three adult shrimp (30 animals in total). Animals in the test group were exposed to acoustic signals [a linear sweep from 10 to 200 kHz lasting 1 s, with a sound pressure level between 138 and 157 dBrms (re 1lParms)] for 45 min. Behavioral parameters such as, total acoustic signals emitted, movement velocity, angular velocity, distance moved, no. of fights, encounters, tail flip and haemolymphatic parameters such as pH, osmolarity, protein concentration and enzyme activities (alkaline phosphatase, esterase and peroxidase) were evaluated. Exposure to the acoustic signal produced significant changes on the specie's total acoustic signals emitted and on number of fights. Enzyme activities show significant changes, with significantly lower values in stressed animals. These results suggest that high-frequency stimuli induce both a behavioural and physiological response, thus suggesting that acoustic stress may have an effect on the species.

ACUTE EXPOSURE OF *ARTEMIA SALINA* TO TIO_2 BROOKITE/CEO_2 NANOPARTICLES

S. Indelicato[1], R. Pecoraro[1], M. Contino[1], G. Ferruggia[1], E.M. Scalisi[1], S. Ignoto[1], G. Coco[1], G. Giuffrida[1], F. Coppa[1], R. Fiorenza[2], S.A. Balsamo[2], A. Salvaggio[3], M.V. Brundo[1]

[1]Department of Biological, Geological and Environmental Sciences, University of Catania, Catania; [2]Department of Chemical Sciences, University of Catania, Catania; [3]Experimental of Zooprophylactic Institute of Sicily "A. Mirri", Palermo, Italy
E-mail: stefaniaindelicato93@gmail.com

Although nanotechnologies play an increasingly important fundamental role today, exposure to nanoparticles, both intentional and unintentional, and their subsequent dispersion in the external environment, seems to be the subject of great interest due to the considerable impact that these nanomaterials can have on health and the marine ecosystem. Due to their distinctive physicochemical and electrical properties, they are widely used in different sectors such as electronics, biotechnology, aerospace engineering and are of great importance in the development of new nanodevices used in numerous physical, biological, biomedical and pharmaceutical applications.[1,2,3] However, depending on the size, concentrations

and timing of exposure, they can be responsible for deleterious effects in both aquatic and terrestrial organisms. In the literature, it is reported that numerous types of NPs are able to overcome certain biological barriers by exerting their effects on different organs. In this study, the effects of TiO2-Brookite NPs combined with CeO_2-NPs, were analyzed. *Artemia salina* nauplii were exposed to different concentrations of TiO_2 Brookite/CeO_2 and their effects on viability at 24 h and 48 h were evaluated. After 48 h of exposure, susceptibility to oxidative stress and induction to the apoptotic process were assessed. Although acute exposure does not result in a statistically significant reduction in viability, the same results, combined with those obtained following qualitative analyses of ROS and apoptosis, could suggest that chronic exposure to Brookite/CeO_2 NPs could have a negative impact on the environment and human health.

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USE OF *ARTEMIA SALINA* IN TOXICITY STUDIES OF NANOMATERIALS

M.V. Brundo[1], R. Pecoraro[1], E.M. Scalisi[1], S. Indelicato[1], G. Giuffrida[1], F. Coppa[1], G. Coco[1], A. Salvaggio[2]

[1]Department of Biological, Geological and Environmental Science, University of Catania, Catania; [2]Exp. Zooprophylatic Inst. of Sicily "A. Mirri", Palermo, Italy
E-mail: mariavioletta.brundo@unict.it

Animal models are employed for *in vivo* assays of nanomaterials, because they allow to reconstruct the typical routes of exposure of organisms to nanostructured materials (pulmonary, epidermal and oral routes). However, the ethicality of *in vivo* testing has led to the use of alternative assays conducted *in vitro*, but even in this case the problem of costs and complexity of the tests remains. Therefore, a good compromise is the use of the marine crustacean *Artemia salina* as a model organism. Toxicity tests on *Artemia salina* are widely used in toxicological research, because they are cheaper, easy and quick compared to *in vivo* and *in vitro* traditional tests.[1] In this work, *Artemia salina* were used to evaluate the acute effects (24-48 h) of MoS_2 powders (Sigma Aldrich 90nm). We have selected the nauplii in the first larval stage (instar I) of *Artemia salina* and exposed them to different solution of MoS_2 (0.5 mg/mL; 0.05 mg/mL; 0.005 mg/mL). We have set up a multi well plates, with ones nauplius per well, for each MoS_2's solution. A control plates have been also included. Until the end of the test, the nauplii were observed through a binocular microscope to record the number of immobile. The results have shown a very low toxicity of all MoS_2 solution, even if the presence of a dark strip inside the gut highlighted the ability of nauplii to ingest MoS_2.

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MORPHOLOGICAL CONDITIONS OF MUSSEL GONADS AFTER EXPOSURE TO POLYSTYRENE MICROPLASTICS ALONE AND CONJUGATED WITH BISPHENOL A OR CADMIUM

T. Chianese, L. Rosati, S. Vorzitelli, V. Paturzo, A. Locascio, M. Sirakov, R. Scudiero

Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: teresa.chianese2@unina.it

In the marine environment, plastic contamination is one of the major contemporary pollution problems. Physical and chemical factors such as temperature wave and photo-oxidation cause the fragmentation process and the formation of microplastics (MPs). The small particle sizes and their increased bioavailability[1] led to the accumulation of MPs in a wide variety of environments at different trophic levels.[2] In addition to their intrinsic toxicity, MPs, due to their high surface area/volume ratio and hydrophobicity, can interact with and absorb other molecules/particles present in the environment, acting as vectors and releasing them at new sites, as well as in living organisms. This effect, called *Trojan Horse*, is based on MPs features (composition, size, shape, color, functional groups). Our research is aimed to verify the cytotoxic effects of polystyrene (PS) microplastics alone and conjugated with bisphenol A (BPA) on the gonads of *Mytilus galloprovincialis*, a good bioindicator of the marine environment.[3] In the preliminary study presented here, mussels were exposed to PS (5 µm) alone and conjugated with BPA for 48 and 72 h at two different concentrations (0.5 µg/mL, 1 µg/mL). Histological analysis showed that treatment with PS alone and conjugated caused structural alterations in ovarian follicles and sperm cysts, in a dose-dependent manner; no differences were observed between the different particles. At the highest concentration, the ovary is characterized by mostly degenerated oocytes; the testis shows the disorganization of germ cells within the cysts. We also recorded the presence of immune cell infiltrates in the connective tissue of all the treated specimens. These preliminary data suggest that MPs alone and conjugated may have a toxic effect on mussels reproduction. Furthermore, biochemical and molecular analyses will be needed to verify whether the conjugated MPs can act as a Trojan Horse in this organism.

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BENZODIAZEPINE DELORAZEPAM INTERFERENCE WITH EARLY *PARACENTROTUS LIVIDUS* DEVELOPMENT

I. Sgariglia[1], L. Viscovo[1], S. Chirullo[1], R. De Rosa[1], P. Denre[1], A. La Pietra[2], C. Fogliano[3], C.M. Motta[1]

[1]Laboratory of Reproductive Toxicology; [2]Laboratory of Cytochemistry and Histochemistry; [3]Laboratory of Ultrastructural Morphology, Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: mottacm@unina.it

Pharmaceutical active compounds and benzodiazepines, in partic-

ular, are discharged in aquatic environments at ng to µg/L concentrations[1,2] and their interference with aquatic flora and fauna[3,4,5] is becoming a matter of emerging concern. This work assessed the disrupting effects of delorazepam on sea urchin *Paracentrotus lividus* development. To this purpose, three near-future concentrations (1, 5, and 10 µg/L) of delorazepam were used in four parallel experiments: to 1) pre-treat eggs or 2) sperm, or to treat 3) gametes at fertilization, or 4) fertilized eggs. The endpoints considered were fertilization rate, percentage of normal or altered morulae and plutei. The presence and localization of carbohydrate residues on plutei were also determined by staining with a panel of fluorescent lectins. Results indicate that delorazepam profoundly interferes with fertilization and development, significantly increasing the percentage of altered plutei. The effects are exerted mainly on sperm and sperm/egg interaction.

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COMPARATIVE COMPOSITION AND DISTRIBUTION OF MUCINS IN THE MANTLE EDGE OF BIVALVES

M.V. Guglielmi, D. Semeraro, M. Marashi, D. Mentino, M. Mastrodonato, G. Scillitani

Department of Biosciences, Biotechnology and Environment, University of Bari Aldo Moro, Bari, Italy
E-mail: marco.guglielmi@uniba.it

Bivalves are widely used for environmental[1] and contamination studies.[2] Some pollutants have been shown to interfere with the quantity and quality of mucus produced by their epithelia such as gills and feet.[3] The mantle tissues are among those most exposed to contaminants, with severe consequences on several functions. Among them, quali-quantitative alterations in mucin production may result in the expression of glycans in mucous secretion, lowering acidity and raising mucus viscosity, with implications for animal health. In this contribution, we compared the mantle edge mucus composition of three commercially important species, the mussel *Mytilus galloprovincialis* (MG) and the Veneridae clams *Chamelea gallina* (CG) and *Ruditapes philippinarum* (RP). MG and RP specimens from Taranto, as well as CG from Margherita di Savoia, were routinely fixed in paraffin and cut into 5 µm thick sections. Mucocytes distribution and secretions were characterized by standard histochemical techniques, such as PAS, Alcian Blue pH 2.5 and HID, and FITC-conjugated lectins histochemistry such as PNA, SBA, WGA, ConA, UEA and LTA. As in many bivalves, MG has a mantle edge with three folds. Small and superficial mucocytes are preferentially place on the inner fold and secrete an acidic sulphate mucus with mannosylated residues. Circulating cells interfere with the results of other lectins. In the two Veneridae instead, the mantle edge has four folds. The mucocytes are large, even deep, with distinct morphologies in the different folds. They appear to produce a considerably more structured mucus, always sulphate, which binds to most of the lectins except for UEA and LTA. The majority of the residues in CG are galactosylated and mannosylated. RP has similar glycosylation patterns, with galactosaminylated and mannosylated residues predominating. The current study adds to our understanding of mucin secretion in edible bivalves and can be used as a reference for ecotoxicological monitoring using these species as models, also for human health.

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TOXICOLOGICAL EVALUATIONS OF GLYPHOSATE IN ZEBRAFISH EARLY-LIFE STAGE

S. Zugaro[1], A. Iannetta[2], L. Della Salda[3], M. Massimini[3], G. Caioni[1], G. Angelozzi[1], A. Cimini[1], M. Perugini[1], E. Benedetti[1]

[1]Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila; [2]Department of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo; [3]Department of Veterinary Medicine, University of Teramo, Teramo, Italy
E-mail: elisabetta.benedetti@univaq.it

Glyphosate is now considered the most widely used herbicide in the world. It is a systemic, non-selective, post-emergency herbicide and it operates *via* the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme. This enzyme is critical in the shikimate pathway. Its use in agriculture determines the destruction of unwanted weeds. The high persistence and its widespread determine the presence of traces of this herbicide increasingly frequently in soil, water, and air, as well as in food,[1] and this becoming a growing concern for human health. A distinctive feature of some of these water environments, particularly those highly polluted, is the low water oxygen concentration. Anyway, many studies about hypoxia did not associate the effects of hypoxia when fish is exposed to contaminants.[2] For this reason, the present study aimed to evaluate the glyphosate potential effects on the zebrafish early-life stages of development in hypoxic conditions. To reproduce the environmental conditions glyphosate was associated with hypoxia induced by $CoCl_2$. The research focused on the evaluation of glyphosate acute toxicity by the Fish Embryo Acute Toxicity (FET) tests and on the evaluation of oxidative stress.

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TOXICOLOGICAL EFFECTS OF MICROPLASTICS ON ZEBRAFISH EARLY DEVELOPMENTAL STAGES

A. La Pietra, D. Lucariello, C.M. Motta, I. Ferrandino

Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: alessandra.lapietra@unina.it

In recent years, scientific research has focused on microplastics (MPs) and the associated effects of their massive diffusion. MPs are found in different environmental matrices, in marine and freshwater ecosystems.[1] Plastic polymers are considered toxic materials,[2] as vectors of microorganisms and other potentially toxic chemicals.[3] MPs could negatively affect the environment, organisms and human health3 but little is known yet.[4] *Danio rerio*, commonly known as zebrafish, is considered an excellent model

organism for ecotoxicological studies. Its embryos are transparent in the early stages of development making it possible to highlight malformation and disorders.[2,5,6] In this study, zebrafish embryos were used to evaluate the possible toxic effects of polystyrene MPs of 1 μm and 3 μm diameter. The concentrations of 0.01, 0.1, 1.0, and 10.0 mgL^{-1} were tested, and the embryos were monitored at 24, 48 and 72 h. Nile Red staining showed that MPs of both sizes enter and accumulate in the embryos and that the effects are more evident at the higher concentrations. Toxicity tests of MPs' exposure demonstrated no mortality and no alteration in hatching timing at any concentrations. On the contrary, higher concentrations (1.0 and 10.0 mgL^{-1}) accelerate the heart rate frequency and cause tail alterations, pericardial oedema and yolk sac deformity. The Acridine Orange staining highlighted the presence of apoptotic cells in the head and tail, at 72 h at the 10.0 mgL^{-1} exposure. No difference was found between the two sizes of microplastics, and both resulted toxic to the zebrafish embryos' development. Future investigations will lead to a better understanding of molecular mechanisms at the base of these results and the effects of long-term MPs exposure.

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MICROPLASTICS AND NANOPLASTICS POLYSTYRENE IMPAIR DIGESTIVE AND BYSSUS GLANDS IN *MYTILUS GALLOPROVINCIALIS*

R. Romano[1,2], G. Maisto[1], L. Rosati[3], C.M. Motta[3], F. Ferrigno[1], M. Russo[1], S. Belardo[1], R. Rozza[1], M. Karam[1], R. Sandulli[1], G.F. Russo[1,2], P. Simoniello[1,2]

[1]Department of Science and Technologies, University of Naples "Parthenope", Naples; [2]International PhD Programme UNESCO Chair "Environment, Resources and Sustainable Development; [3]Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: gelsomina.maisto001@studenti.uniparthenope.it

Plastic is the most prevalently used material in our modern society; all socioeconomic activities are supported by plastic production that has exponentially increased in the past 70 years. More than 94% of plastics currently present in oceans are represented by Microplastics (MPs) (<5 mm), further degraded into Nanoplastics (NPs) (< 100 nm).[1] To understand how MPs and NPs interfere with marine organisms' life, *Mytilus galloprovincialis* was used in the present study as a model organism.[2,3] In this work the effects of polystyrene on the fitness and behaviour of the bivalve mollusc model were detected, analysing the digestive glands and byssus apparatus respectively involved in the feeding process and adhesion to the substrate, conferring resistance to waves. Mussels were exposed to polystyrene (5 or 0.1 μm) for 1, 3 and 11 days at frequent environmental concentrations.[4] Organs were processed for light microscopy. Results showed that MPs and NPs affect the digestive gland structures in the tubules and ducts organization, interfering with the proliferative activity and inducing collagen deposition within the digestive tubules. Impairment in the byssus glands structures and altered byssal thread production were detected. Further investigations are ongoing to better clarify these aspects. In conclusion, MPs and NPs impair the feeding activity and substrate adhesion of *Mytilus galloprovincialis* with consequences on its fitness. Reducing MPs and NPs contamination is a crucial aspect to recover and defend the ecosystems.

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EFFECTS OF AN EXTENSIVELY USED UV-FILTER (OXYBENZONE) ON THE ELIMINATION OF LIPOPHILIC TOXINS IN THE CLAM (*DONAX TRUNCULUS*)

F. Brandão[1], M.J. Botelho[2,3], S.D. Cruz[4], S. Joaquim[3,5], D. Matias[3,5], M. Candeias[2], M. Castro[5], M. Gaspar[5,6], M. Pacheco[1], P. Pereira[1]

[1]Department of Biology and CESAM, University of Aveiro, Aveiro, Portugal; [2]IPMA, Algés, Portugal; [3]CIIMAR, University of Porto, Matosinhos, Portugal; [4]IENFOCHEM grp. Environ. Chemistry Dept., Inst. of Environ. Assess. and Water Res.-Severo Ochoa Excellence Cent., Spanish Council for Sci. Res. IDAEA-CSIC, Barcelona, Spain; [5]Portuguese Institute of the Sea and the Atmosphere (IPMA), Olhão, Portugal; [6]CCMAR, University of Algarve, Campus de Gambelas, Faro, Portugal
E-mail: fatimabrandao@ua.pt

The capture of *Donax trunculus*, a clam with high economic value in southern Europe, is often interdicted due to the presence of lipophilic toxins [okadaic acid (OA) and dinophysistoxin-2 (DTX-2)]. In southern Portugal, interdiction periods for *D. trunculus* capture have been increasing, mainly in summer. It can be hypothesized that enriched levels of contaminants, such as UV filters, in coastal waters may overload toxin elimination pathways (OA and DTX-2), inhibiting *D. trunculus*'s ability to metabolize these toxins. Clams naturally contaminated by OA and DTX-2 (111 μg equivalents of OA per kg of shellfish) were exposed to 5 μg/L of oxybenzone (Oxy) for 7 days. Control clams were naturally contaminated with OA and DTX-2. Clams were sampled before exposure (T0) and 4 and 7 days after exposure to Oxy (E4 and E7) to assess OA and DTX-2 values in edible tissues, as well as for the evaluation of biochemical endpoints (GST, GSHt, GR, GPx, SOD, LPO). A reduction of both lipophilic toxins values was observed at E4, with slightly higher elimination rates for Oxy-exposed clams. Although these clams exhibited higher GR than the controls, it is unlikely that this change is related to Oxy exposure. No significant decreases in OA values were observed between conditions at E7, but the decrease of DTX-2 values was higher under Oxy exposure. Data on OA and DTX-2 toxicokinetics and biochemical endpoints revealed that Oxy exposure did not compromise the metabolism of those toxins.

EXPLORING RETINOIC ACID ROLE IN CRINOID EMBRYOGENESIS

S. Mercurio[1], G. Blumer[1], G. Scarì[2], R. Pennati[1]

[1]Department of Environmental Science and Policy, University of Milan; [2]Department of Biosciences, University of Milan, Italy
E-mail: sil.mercurio@gmail.com

Retinoic acid (RA) is a Vitamin A-derived molecule which plays fundament roles in development.[1] RA signalling was initially considered a chordate innovation, but, the identification of key components of RA machinery in several invertebrates has definitely demonstrated that the appearance of RA pathway predated the origin of bilaterians.[2,3] Nevertheless, the physiological roles of retinoids in invertebrates are still poorly understood and novel biological functions continue to emerge.[1,4] One of the crucial role of RA is its involvement in the determination of embryo body axis and the regulation of *Hox* genes.[5] In this context, echinoderms are of great interest from an evolutionary point of view as they are marine deuterostomes with a secondary pentameral symmetry. Larvae display a bilateral body plan which is lost during metamorphosis when the typical adult radial body develops. Only a few research explored RA functions in echinoderm development: although RA genetic toolkit is present in the genome of several model species, RA exposure induced only minor effects during sea urchin embryogenesis while metamorphosis was strongly affected by the treatment in asteroids and crinoids.[6,7,8] We started to explore the role of RA in crinoid embryogenesis by exposing embryos of the Mediterranean feather star *Antedon mediterranea* to different concentrations of RA. Preliminary results suggested that RA affects larvae morphology and prevents the onset of metamorphosis. Additionally, to provide a comprehensive understanding of the role of RA in crinoid development, we exposed hatching larvae to the same concentrations of RA and described the effects using different microscopy techniques. Given the crucial phylogenetic position of crinoids, our results are critical in elucidating the role of RA in echinoderm embryogenesis and confirming its involvement in metamorphosis.

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DNA DAMAGE BY POLYSTYRENE MICROPLASTICS IN ZEBRAFISH

F. Mottola[1], M. Carannante[1], M. Santonastaso[2], R. Scudiero[3], L. Rocco[1]

[1]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "Luigi Vanvitelli", Caserta; [2]Department of Woman, Child and General and Special Surgery, University of Campania "Luigi Vanvitelli", Naples; [3]Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: maria.carannante@unicampania.it

In recent years, plastic pollution has become global environmental concern affecting both terrestrial and aquatic environments.[1,2] Microplastics (MPs) derived from the degradation of plastic through physical-chemical processes, can be ingested by organisms and reach humans through the food chain. Although the mechanism of action and the health impact on the of exposed organisms are not yet fully understood, the literature data confirm deleterious health consequences following exposure to different types of microplastics.[3,4] The aim of this research was to evaluate the *in vitro* genotoxic effects of polystyrene MPs on *Danio rerio* cells using RAPD-PCR, to quantify the genomic template stability (GTS), TUNEL reaction to evaluate MP-induced DNA fragmentation (DFI) and DCF assay to highlight a possible ROS-dependent mechanism of damage. Zebrafish blood cells were exposed to MPs (105 µg/mL) for 30, 60 and 90 min. The RAPD- PCR results showed a 20% reduction in GTS after 30 and 60 min of MP exposure reaching up to 40% after 90 min. Similarly, a time-dependent increase in MP- induced DFI was observed, with DFI reaching values up to 37% for the maximum exposure time. Furthermore, the presence of intracellular ROS was highlighted for the longer exposure times. These results confirm the harmfulness of MPs, in particular their ability to interfere with genetic material causing apoptosis *via* oxidative imbalances. Further *in vitro* and *in vivo* studies evaluating tbe bioaccumulation processes will be needed to establish the mechanisms underlying the MPs damage.

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ENVIRONMENTAL CHANGES AFFECT TRANSPOSON EXPRESSION IN ZEBRAFISH (*DANIO RERIO*)

C. Arena[1], L. Fanti[1], C. Cioni[1], P. Franchini[2], M. Toni[1]

[1]Department of Biology and Biotechnologies "Charles Darwin", Sapienza University, Rome; [2]Department of Ecology and Biology (DEB), Tuscia University, Italy
E-mail: arena.1689984@studenti.uniroma1.it

Human activities alter the environment through the emission of chemical substances that pollute the soil and the waters or contribute to the global warming. Growing evidence suggests that environmental stressors can influence the expression and activity of transposable elements (TEs).[1] TEs are DNA repetitive sequences able to move from one location to another within the genome. TEs activity can be mutagenic in organisms including humans, but it may also act as driving force at evolutionary level. The zebrafish is a good study model in many research areas, including molecular biology and ecotoxicology. Our research group demonstrated that environmental temperature alteration strongly impacts the brain proteome and behaviour of zebrafish[2,5] and Chen *et al*. observed that cold temperature induces retrotransposition in zebrafish.[6] Bioinformatics analysis of transcriptomes is a useful tool to identify variations in TEs expression in teleosts.[7] In this study, we performed an RNA-seq data analysis to evaluate the effect of thermal, polluting and pharmacological treatments on TEs expression in zebrafish. Briefly, transcriptomes available in databases were processed by FastQC, Trimmomatic and Cutadapt software for sequence quality control, by HISAT2 to align the reads, and by TEcount to quantify TEs expression. Finally,

DESeq2 was used to estimate the differential expression of TEs. Results show that the presence of pesticides, nanoparticles and drugs in the water, temperature alteration and hypoxia lead to an altered TEs expression in zebrafish.

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GENOTOXIC EVALUATION IN ZEBRAFISH OF QUINOIN, TYPE 1 RIBOSOME-INACTIVATING PROTEIN FROM QUINOA SEEDS

F. Mottola[1], M. Carannante[1], N. Landi[1], S. Ragucci[1], R. Scudiero[2], M. Della Corte[1], D. Paciolla[1], A. Di Maro[1], L. Rocco[1]

[1]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "Luigi Vanvitelli", Caserta; [2]Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: filomena.mottola@unicampania.it

Nowadays, functional foods have greatly increased attention thanks to the numerous benefits for human health. Among them, quinoa contains all essential amino acids and minerals, for which is particularly suitable for consumption. However, quinoin (~30-kDa), a toxic enzyme classified as ribosome inactivating protein (RIP, EC: 3.2.2.22)[1], recently found in quinoa seeds (*Chenopodium quinoa* Wild), exhibits *in vitro* cytotoxic action towards both normal fibroblasts and keratinocytes[1] and several tumour cell lines.[2] In this scenario, our study aims to evaluate quinoin genotoxicity on zebrafish specimens after intraperitoneal route administration of three different quinoin amounts (1- 5-10 μg) for 15 and 30 days of treatment by means: TUNEL reaction, RAPD-PCR and DCF assay. The results obtained showed that quinoin cause zebrafish genome damage, in terms of DNA fragmentation, genomic instability and oxidative stress for all exposure times. The interesting data emerging from this study is a lower percentage of damage at longer quinoin treatment compared to shorter ones. This result could indicate the activation of detoxifying and/or repair mechanisms and/or a loss of protein activity by enzymatic digestion in the gastrointestinal tract since digestibility of quinoin by pepsin/trypsin *in vitro* digestion system has been ascertained[1]. Overall, quinoin can induce genotoxic damage to the zebrafish genome acting through ROS formation. Thus, our data suggest that the presence of quinoin in quinoa seeds could be very harmful if this pseudocereal is consumed with appropriate cooking, considering the melting temperature (Tm= 70°C) of quinoin[1]. Further studies are needed to investigate DNA damage response pathways to clarify the effective quinoin harmfulness.

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BENEFICIAL BACTERIA TO COUNTERACT PFOA TOXICITY ON *DANIO RERIO* DEVELOPMENT

C. Giommi[1,2], M. Lombó[1,2,3], F. Francioni[1], L. Bevilacqua[1], H.R. Habibi[4], F. Maradonna[1,2], O. Carnevali[1,2]

[1]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy; [2]INBB - Interuniversitary Consortium of Biosystems and Biostructures, Rome, Italy; [3]Department of Molecular Biology, University of León, León, Spain; [4]Department of Biological Sciences, University of Calgary, Calgary, Canada
E-mail: c.giommi@pm.univpm.it

The surfactant Perfluorooctanoic acid (PFOA), a contaminant of emerging concern, is environmentally ubiquitous and its resistance towards biological degradation allows its bioaccumulation within organisms. Previous studies demonstrated that PFOA affects *Danio rerio* embryo development affecting heartbeat rate and locomotor behaviour.[1] Nevertheless, it is known that probiotics in zebrafish ameliorate the host health and improve embryo development and ossification.[2] Based on previous results, the present study aims at determining the role of *Bacillus subtilis natto* to mitigate the PFOA developmental toxicity during zebrafish early development. Control and exposed larvae (50 and 100 mg/L)[3] were reared starting from hatching until 21 days post fertilization (dpf) with or without *Bacillus subtilis natto* dietary administration (10^7 CFU/larvae/day). At 21 dpf, a decrease of standard length associated to an increase of the head length was observed in fish exposed to PFOA. Noteworthy, probiotic when co-administered with PFOA rescued both body and head length to control levels. Considering ossification, the lowest concentration resulted the most harmful increasing the rate of craniocephalic malformations and eye diameter. The ability of the probiotic to mitigate the toxic effects of PFOA was even more evident in larvae exposed to the highest dose of contaminant. In order to understand these phenomena, expression of genes involved in growth (*mstn, igf1, igf2* and *thrα*) and ossification (*col10a1a, runx2b, cyp26b1* and *spp1*) was analysed at 7, 14 and 21 days. Both doses altered thyroid function at 7 dpf while only the lowest one affected ossification at 21 dpf. At this last stage, probiotic antagonize PFOA effects, suggesting its suitability as tool to counteract its endocrine disrupting activity.

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TEMPOL-METHOXYCINNAMATE, AN ENVIRONMENTALLY-FRIENDLY UV FILTER? EVIDENCE FROM ZEBRAFISH EARLY DEVELOPMENT

F. Sella[1], E. Damiani[1], M. Marasco[1], P. Astolfi[2], R. Galeazzi[1], O. Carnevali[1], F. Maradonna[1]

[1]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona; [2]Department of Science and Engineering of Materials, Environment and Urban Planning, Polytechnic University of Marche, Ancona, Italy
E-mail: f.sella@pm.univpm.it

In the last years, the use of ultraviolet (UV) organic filters in personal care products has increased due to the growing need of preventing skin damage caused by UV radiation overexposure. Therefore, the massive use has resulted in their presence in most aquatic matrices, causing a series of adverse effects in organisms.

To date, octyl methoxycinnamate (OMC), has been used worldwide in over 90% of cosmetic products, however its endocrine disrupting effects have been documented *in vitro* and *in vivo* studies.[1,2] Following Hawaii's recent ban on selling sunscreens containing OMC, the cosmetic industries have been encouraged to review their formulations, looking for new ingredients to offer safer sunscreens. In this study, the first *in vivo* insights on the effects of Tempol-methoxycinnamate (TMC), a recently synthesized OMC derivative,[3] on zebrafish, *Danio rerio*, early development is reported. Embryos were exposed to TMC and OMC in the range of OMC concentrations detected in the environment[4] and their effects were analyzed and compared to view TMC as possible candidate alternative to OMC. Results pinpointed that TMC exposure did not affect the hatching rate nor morphometric growth parameters and ossification process with respect to control fish. Differently from OMC, TMC does not induce endoplasmic reticulum (ER) stress response, as suggested by *eif2ak3, ddit3, nrf2,* and *nkap* mRNA levels and as emerged by measuring *ar* transcript and Erα protein levels, has scarce hormone-like activity. This last aspect was enforced by molecular docking analysis, which showed a hormone-like activity for OMC but not for TMC. Moreover, TMC does not affect apoptosis which has a key role in embryoshaping during early development. In conclusion, this study indicates the suitability of TMC as promising and safer UV filter.

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AN INSIGHT INTO THE HAZARDOUS EFFECTS OF GLYPHOSATE DIETARY DOSES ON ZEBRAFISH MALE REPRODUCTION

M. Lombó[1,2], C. Giommi[1,2], R. Bhatt[3], M. El Kamouh[4], H.R. Habibi[5], F. Maradonna[1], O. Carnevali[1]

[1]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy; [2]Department of Molecular Biology, University of León, León, Spain; [3]Navrachana University, Vadodara, Gurajat, India; [4]INRAE, Fish Physiology and Genomics, Rennes, France; [5]Department of Biological Sciences, University of Calgary, Calgary, Canada
E-mail: mloma@unileon.es

Glyphosate (GLY) is one of the world's leading agrochemicals since it is widely applied for weed control and desiccation. In Italy, although the use of GLY for gardening and pre-harvesting purposes has been banned, this compound is still present in edible products due to its application during harvest and threshing as well as to the import of wheat from countries that are more permissive to GLY use.[1] Considering that growing evidence reports the endocrine-disruptive and epigenotoxic effects of this chemical,[2] this study aims to better understand its impact on both reproductive health and offspring development. To shed some light on the effects of environmentally relevant doses of GLY on spermatogenesis and breeding capacity, zebrafish adult males were dietary exposed to three different doses of GLY: 0.5, 5, and 50 mg/kgbw/day (all of them considered by the EFSA below the threshold of the observable adverse effect level dose)[3] for three weeks. The results showed that the lowest dose decreased the gonadosomatic index, altered the gonad architecture, and triggered changes in histone acetylation depending on the stage of germ cell maturation. These modifications seem to be associated with a disruption of the endocannabinoid system. Despite the fact that few effects were found regarding the medium dose, the highest one dramatically impaired male breeding capacity by modifying not only the testicular architecture but also the transcription of genes involved in steroidogenesis (*star, cyp11a1, cyp17a1*) and increasing the apoptosis within the testes. Altogether, these data support the need of a deeper revaluation of the GLY safety standards by the agencies and lawmakers at national and European level.

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RACK1 IS INVOLVED IN EMBRYONAL GENES EXPRESSION IN ZEBRAFISH HEART HYPERTROPHY

M. Ceci[1], D. Bonvissuto[2], F. Papetti[1], R. Marangon[1], F. Silvestri[1], C. Sette[2], E. Catalani[3], D. Cervia[3], R. Gornati[4], N. Romano[1]

[1]DEB, Tuscia University, Viterbo; [2]Department of Neuroscience_Human Anatomy, University of Sacred Heart, Rome; [3]DIBAF, Tuscia University; [4]DBSV - Insubria University, Varese, Italy
E-mail: nromano@unitus.it

Cardiac hypertrophy is a frequent cause of heart failure. The zebrafish heart culture in *ex-vivo* is a powered tool to study cardiac hypertrophy[1,2] because it is possible to control the activation of critical genes and thus test drug therapies before the *in vivo* tests.[2,3] Previous observations have demonstrated the embryonal gene re-expression in cardiac cells under hypertrophic or growth factors cocktail (GFs) stimuli.[2,4] The RACK1 protein is homologous to the β subunit of G-proteins and is involved in transcription/translation gene expressions.[5] Since a role of the RACK1 protein has been suggested in mouse models of cardiac hypertrophy,[6] here we investigated its involvement in the embryonal gene expressions in our zebrafish *ex-vivo* model, by using Phenylephrine (PE) or GFs as pro-hypertrophic stimuli. The role of RACK1 in the inhibitory action of Blebbistatin (BL) was studied as well. Indeed, has an essential role in blocking the Ca^{2+} waves thus inhibiting some of the transductional actions of PE[3]. PCR and Immunoblot analyses confirmed the upregulation of RACK1 in the PE or GFs-treated groups. The administration of BL counteracted the PE-induced hypertrophy and RACK1 expression. Immunohistochemical analyses in the cardiac cells showed the co-localization of RACK1 and the different embryonal genes, *i.e.* GATA4, WT1, and NFAT2. of interest, cultured cells of zebrafish heart activated to express embryonal genes by GFs treatment[3] and transfected with RACK1+-plasmid, displayed higher levels of embryonal genes. Taken together, these results evidenced the involvement of RACK1 in the embryonal gene expression during cardiac hypertrophy. Further studies will be addressed to investigate the mechanisms responsible of this effect.

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IN VIVO PRO-ANGIOGENIC POTENTIAL OF MESENCHYMAL STEM CELL- CONDITIONED MEDIUM PRODUCED UNDER NORMOXIC AND HYPOXIC CONDITIONS

L. Barone[1], F. Rossi[1], R. Papait[1], N. Romano[2], M. Raspanti[3], P. Zecca[3], A. Bruno[1,4], L. Valdatta[1], G. Bernardini[1], R. Gornati[1]

[1]Department of Biotechnology and Life Sciences, University of Insubria, Varese; [2]Department of Ecological and Biological Sciences, Tuscia University, Viterbo; [3]Department of Medicine and Surgery, University of Insubria, Varese; [4]Laboratory of Innate Immunology, IRCCS MultiMedica, Milan, Italy
E-mail: rosalba.gornati@uninsubria.it

Over the past decades, the role of human adult mesenchymal stem cells (hMSCs) has been widely investigated for regenerative medicine applications. However, due to the many limitations associated with the transplant of viable cells, the research focus has shifted to their conditioned medium (CM). There is, in fact, a growing body of evidence suggesting the high capacity of hMSC-CM in stimulating tissue regeneration and angiogenesis. Among hMSCs, adipose-derived stem cells (hASCs) and their pro-angiogenic potential have been evaluated;[1] conversely, very little is known about dental pulp stem cells (hDPSCs). In this scenario, this work aims to evaluate the potential of hMSC-CM in promoting angiogenesis, which is necessary for tissue regeneration. Both hASCs and hDPSCs have been cultured, in starvation, under normoxic (20% O_2) and hypoxic (2% O_2) conditions for 72 h, after which their CMs have been collected and concentrated. To evaluate their *in vivo* potential to stimulate angiogenesis, the Ultimatrix sponge assay was performed. Briefly, the hMSC-CMs were associated with the Ultimatrix scaffold and injected in 7-week-old BALB-C nude athymic mice for 6 days. Subsequently, the scaffolds were removed and analyzed by FACS, optical and scanning electron microscopy to assess the vascularization. The results obtained showed the presence of newly-formed vessels inside the scaffolds, thus demonstrating the efficiency of the hMSC-CMs in inducing *in vivo* angiogenesis and showing that hDPSC-CM resulted as efficient as hASC-CM. Moreover, hypoxic conditions seem to be able to improve this cell-free system, thus representing an effective tool worth investigating for regenerative medicine applications.

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THE ROLE OF TAZ IN THE EPIGENETIC MAINTENANCE OF CARDIOMYOCYTE HOMEOSTASIS

C. Pagiatakis[1,2], S. Serio[1,2], J. Laura Francés[2], E. Musolino[2], L. Papa[2], A. Felicetta[2], G. Condorelli[2], R. Papait[1,2], R. Gornati[1], G. Bernardini[1]

[1]Department of Biotechnology and Life Sciences, University of Insubria, Varese; [2]IRCCS Humanitas Research Hospital, Rozzano, Italy
E-mail: christina.pagiatakis@uninsubria.it

Cardiomyocytes respond to stress through hypertrophic growth, the hallmarks of which are an increase in their mass and size, modification of their shape, and activation of the foetal cardiac gene program. The epigenetic mechanisms governing cardiac gene expression regulating cardiac development and homeostasis are still not fully understood. The Hippo signalling pathway is a critical pathway involved in development, organ size control and stem cell fate, however the specific role of the main effectors YAP and TAZ remains to be fully elucidated in the heart. Although recent studies have revealed that chronic suppression of the Hippo pathway is detrimental to the heart during pressure overload, the distinct role of TAZ remains elusive. Thus, we aimed to understand the role of TAZ in the maintenance of cardiac homeostasis. We have already demonstrated that activation of TAZ post-stress results in an interaction with MEF2C (myocyte enhancer factor 2C), increasing its transcriptional activity. MEF2C regulates genes necessary for cardiac development and maintains cardiomyocyte function post-natally: aberrant activation of MEF2C disrupts cardiac homeostasis by activating foetal gene expression in the adult heart. We have also previously shown that the histone methyltransferase G9a is necessary for the repression of MEF2C activity in the adult heart, and interestingly, TAZ interacts with G9a and inhibits the repressive effect of G9a on MEF2C, regulating the epigenetic landscape of genes regulated by MEF2C which concurrently carry signature histone modifications due to G9a. Our results strongly suggest that TAZ is responsible for the alteration of cardiomyocyte homeostasis *via* a MEF2C-G9a-dependent mechanism, resulting in perturbations in gene expression patterns during pressure-overload by interacting with, and counterbalancing the effects of G9a on MEF2C. Elucidation of this pathway will significantly improve knowledge of the epigenetic mechanisms governing the gene expression program required to maintain cardiac homeostasis.

BROMODOMAIN AND EXTRATERMINAL DOMAIN (BET) PROTEINS INHIBITION HINDERS GLIOBLASTOMA PROGRESSION BY INDUCING AUTOPHAGY-DEPENDENT DIFFERENTIATION

D. Gargano[1], M. Colardo[1], M. Russo[1], M. Petraroia[1], D. Pensabene[2], M. Segatto[1], S. Di Bartolomeo[1]

[1]Department of Biosciences and Territory, University of Molise, Pesche (IS); [2]Department of Science, University Roma Tre, Rome, Italy
E-mail: d.gargano@studenti.unimol.it; sabrina.dibartolomeo@unimol.it

BET proteins are a family of multifunctional epigenetic readers, mainly involved in transcriptional regulation through chromatin modelling.[1] Transcriptome handling ability of BET proteins suggests a key role in the modulation of cell plasticity both in fate decision and in lineage commitment during embryonic development and in pathogenic conditions, including cancerogenesis.[2] Glioblastoma multiforme (GBM) is the most common and aggressive malignant primary brain tumor and it is characterized by high recurrence incidence and poor prognosis due to the presence of a highly heterogeneous mass of a stem cells with self renewal capacity and maintenance of stemness.[3] In recent years, the epigenetic landscape of GBM has been explored and many epigenetic alterations have been investigated. Among the epigenetic abnormalities, the BET proteins have been found significantly overexpressed in GBM.[4] We recently investigated the effects of BET proteins inhibition on GBM cells reprogramming. We found that the pan-BET pharmacological inhibitor JQ1 was able to promote a differentiation program in GBM cells, thus impairing cell proliferation and enhancing the toxicity of the drug Temozolomide (TMZ).[5] Notably, the pro-differentiation capability of JQ1 was prevented in autophagy-defective models, suggesting that autophagy activation is necessary for BET proteins activity in regulating glioma cell

fate[5]. Given the growing interest placed in epigenetic therapy and the compelling demand for more effective therapeutic strategies, our results support the hypothesis to introduce BET-based approaches in GBM clinical management.

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GLIOBLASTOMA-DERIVED EXTRACELLULAR VESICLES INDUCED MATURATION OF DENDRITIC CELLS: A PROMISING APPROACH FOR IMMUNOTHERAPY

D. Vardanyan[1], I. Efimova[3,4], R. Demuynck[3,4], S.Tacconi[2], D.V. Krysko[3,4], L. Dini[1]

[1]Department of Biology and Biotechnologies Charles Darwin, the Sapienza University of Rome, Rome, Italy; [2]CarMeN Laboratory (UMR INSERM 1060/INRA 1397), Lyon-Sud Faculty of Medicine, University of Lyon, Pierre-Bénite, France; [3]Cell Death Investigation and Therapy Laboratory, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; [4]Cancer Research Institute Ghent, Ghent, Belgium
E-mail: diana.vardanyan@unisalento.it

Glioblastoma is a type of brain cancer that is highly aggressive and difficult to treat. It is characterized by rapid growth and invasion into surrounding brain tissue, making it challenging to completely remove with surgery or treat with chemotherapy or radiation therapy. Antigen-presenting cells (APCs) such as dendritic cells (DCs) play an essential role in activating T cells during the cancer-immunity cycle by capturing, processing, and presenting antigens derived from tumour cells.[1] Multidirectional crosstalk between DCs and tumours is partially mediated by extracellular vesicles (EVs) secreted by both cell types.[2,3] By performing proteomic analysis, we determined the molecular profiles of EVs from U87MG and U373MG human glioblastoma cell lines with and without temozolomide (TMZ). Using confocal microscopy, we confirmed that DCs internalized the EVs. Through flow cytometry, DC maturation status was analyzed. As a result of our study, treatment with EVs derived from temozolomide-treated glioblastoma cells upregulated the expression of the major histocompatibility complex class II (MHC-II) and CD80/86 costimulatory molecules on DCs, and consequently, EVs from TMZ-treated glioblastoma cells may be useful as a therapeutic agent for glioblastoma since they can activate the immune system thereby affect the cancer cells. Overall, the interaction between EVs and DCs in the context of glioblastoma is complex and requires further investigation to fully understand the mechanisms involved. However, targeting this interaction may hold promise for developing new immunotherapeutic strategies for treating glioblastoma.

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CYTOTOXICITY OF EXTRACTS FROM LEAVES AND RHIZOMES OF *POSIDONIA OCEANICA* ON HEPG2 TUMOR CELLS: FOCUS ON AUTOPHAGY AND APOPTOSIS

G. Abruscato, R. Chiarelli, D. Ganci, R. Tarantino, M. Mauro, A. Vizzini, M. Vazzana, V. Arizza, C. Luparello

Department of Biological, Chemical and Pharmaceutical Sciences, University of Palermo, Palermo, Italy
E-mail: giulia.abruscato@unipa.it

Bioactive compounds from aquatic species exert various beneficial effects in humans, thereby being the object of studies as disease prevention/treatment agents or food supplements in a circular economy and waste-recycling context.[1,2] Aqueous extracts from green leaves (GLE) and rhizomes (RE) of the seagrass *P. oceanica*, characterized by phenolic compound and proteomic analyses, had already been shown to impair HepG2 hepatocarcinoma cell viability/motility, mitochondrial function and redox state.[3] Here, we focused onto the impact of apoptosis and autophagy modulation on the cytotoxic effect exerted by GLE and RE IC_{50}. Cell cycle and Annexin V-FITC binding assays proved the apoptosis-promoting effect of both extracts, also confirmed by the activation of caspase-1, -2, -3 (with GLE only) and -6. The intracellular accumulation of AVOs, hallmarks of autophagy, was downregulated by both treatments, more sharply by RE. Cotreatment of GLE, but not RE, with the autophagy-stimulator rapamycin reverted viability decrease suggesting a more extensive RE-induced cell damage. Autophagy and apoptosis were further investigated at the molecular level. Western blot quantitation of LC3, Beclin-1, p62/SQSTM1 and hsp60, markers of autophagy and cytoprotection, and qRT-PCR analyses of the modulation of the expression of *BCL2, BAX, BAD, FOS, JUN* and *DAPK* genes, coding for factors involved in the apoptotic and autophagic processes, were performed on HepG2 cell preparations obtained at different times of exposure. Our cumulative data strongly support the assumption that apoptotic promotion and autophagy downregulation are involved in the *in vitro* anti-liver cancer activity of the two different *P. oceanica*'s phytocomplexes, although to different extents conceivably due to their different compositions. These results prompt further investigation to identify other molecular targets of GLE and RE cytotoxicity and extend the exposure response screening to other human tumoral and normal cell lines.

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OXIDATIVE STRESS AND LIPID ACCUMULATION ASSOCIATED WITH AN *IN VITRO* NAFLD MODEL WERE REDUCED BY OLIVE LEAF EXTRACT

S. Tacconi[1,2], S. Longo[3], C. Moliterni[2], V.M. Paradiso[3], G. Difonzo[4], F. Caponio[4], D.Vergara[3], L. Dini[2,5], A.M. Giudetti[3]

[1]CarMeN Laboratory, University of Lyon, France; [2]Department of Biology and Biotechnologies Charles Darwin, Sapienza University of Rome, Italy; [3]Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy; [4]Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy; [5]Research Center on Nanotechnology Applied for Engineering of Sapienza University of Rome, Italy
E-mail: camilla.moliterni@uniroma1.it

Nonalcoholic fatty liver disease (NAFLD) is defined as the accumulation of intracellular fat in the liver that exceeds 5% of the weight of the liver itself, often resulting from excessive consumption of high-fat-containing foods. Oxidative stress accompanying NAFLD can lead to severe liver diseases, ranging from steatosis to fibrosis and to cirrhosis and eventually to hepatocellular carcinomas. Olive leaf extract (OLE) is a reliable source of polyphenols with antioxidant and hypolipidemic properties that have been successfully used in medicine, cosmetics, and pharmaceutical products. OLE extract produced from agricultural waste by using green solvent, allows for a sustainable approach, and preserves the extract's beneficial properties, representing one of the major challenges of biomedical research. The aim of this study is to evaluate the potential antioxidant and lipid-lowering effect of "green" OLE obtained by a water ultrasound-assisted extraction procedure on HuH7 human liver cells treated with a high amount of free fatty acids (FFA). The main features of FFA treatment, *i.e.* lipid droplets accumulation, modulation of protein expression and oxidative stress were examined. High FFA concentration triggers lipid accumulation and increases hydrogen peroxide levels and reduces the activity of antioxidant enzymes. Coincubation of high FFA with OLE reduced lipid and H_2O_2 accumulation and increased the activity of peroxide-detoxifying enzymes. OLE improved mitochondrial membrane potential and liver parameters by restoring the expression of enzymes involved in insulin signaling and lipid metabolism. Electron microscopy revealed an increase in autophagosome formation in both FFA- and FFA+OLE-treated cells. The study of the autophagic pathway indicated OLE's probable role in activating lipophagy.

EFFECTS OF HYDROGEN PEROXIDE ON APOPTOSIS AND AUTOPHAGY OF MESOANGIOBLAST (C57) CELLS

R. Chiarelli[1], M.M. Barreca[2], F. Geraci[1]

[1]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies; and [2]Department of Biomedicine, Neurosciences and Advanced Diagnostics, University of Palermo, Palermo, Italy
E-mail: fabiana.geraci@unipa.it

One of the limiting factors in the use of stem cells in regenerative medicine is their high mortality rate during the first days post-transplantation. Indeed, the microenvironment within damaged tissues is hostile for stem cell survival and hydrogen peroxide (H_2O_2) may play a relevant role in inducing death of the injected cells. H_2O_2 is well known as a cell damaging agent that is produced during normal cell metabolism of aerobic organisms. The aim of our study was to determine the mechanism of mesoangioblast (C57) cell death after an H_2O_2 treatment. Several assays showed that H_2O_2 induced apoptosis in C57 cells. In particular, FACS analysis with annexin V/PI showed that H_2O_2 induced a time-dependent decrement in A6 viability. Apoptotic cell death was confirmed by the increase in caspases 8, 9 and 3/7 activity after the treatment. The increase in caspase 8 and 9 activity suggested that both apoptotic pathways were activated by H_2O_2. Moreover, apoptosis was proved by TUNEL assay and by measuring mitochondrial membrane potential with the JC-1 dye. Then, we tested whether H_2O_2 is responsible for autophagy activation. To this aim, we evaluated the expression of specific markers such as LC3-GFP (autophagosomes marker) and acridine orange staining of late-stage autophagy (autolysosomes marker). Preliminary data seems to demonstrate that C57 treatment with H_2O_2 induced also autophagy activation and execution. Further analysis will be necessary to investigate the possible interaction between the two pathways. A better understanding of why stem cells die after transplantation will help their use in regenerative medicine.

AIR POLLUTION AND RESPIRATORY VIRAL DISEASES: A CASE STUDY ON THE EFFECT OF PM2.5 AND SARS-CoV-2 IN HUMAN LUNG CELLS

S. Marchetti, M. Gualtieri, C. Bragato, A. Colombo, P. Mantecca
POLARIS Research Centre, Department of Earth and Environmental Sciences, University of Milano-Bicocca, Milan, Italy
E-mail: sara.marchetti1@unimib.it

Airborne pathogens represents a topic of great scientific relevance, especially considering the recent COVID-19 pandemic. Air pollutant such as Particulate Matter (PM) can in fact be associated with an increased incidence of respiratory viral diseases.[1] To provide useful insights into the mechanisms by which PM could be involved into infection, we exposed human lung cells to fine PM ($PM_{2.5}$) and SARS-CoV-2, in order to evaluate different particles cytotoxicological properties and the molecular pathways activated after 72 h of treatment. We also explored the combined effects of PM2.5 and SARS-CoV-2, to verify the possibility that $PM_{2.5}$ may play a role in facilitating SARS-CoV-2 infection. Results demonstrated that inflammation is the key process involved in the cell response to PM2.5 and viral particles, albeit with differences. Interestingly, the harmful effects are increased when SARS-CoV-2 and $PM_{2.5}$ are combined. We also found that PM2.5 induces an over-expression of the angiotensin 2 converting enzyme (ACE2), a preferential entry for the viral particles into respiratory host cells.[2] We then verified the possibility that a sub-chronic exposure to PM could induce a reduction of cellular defenses, thus making people more susceptible to infection. Cells were treated with $PM_{2.5}$ for 72 h, afterward SARS-CoV-2 was added for additional 2 and 24 h. Data showed that pre-treatment with $PM_{2.5}$ facilitates virus entry within the endosomal route already after 2 h of exposure to SARS-CoV-2. We also demonstrated that the cells exposed to PM and successively treated with virus for 24 h, respond with an intensified inflammatory state. Taken together, these results showed that $PM_{2.5}$ appears to facilitate the virus entry in the lung, besides worsening the potency of the inflammatory response to viral infection. The times we are living in, solicit the urgency to carry out a more in-depth investigation, to provide useful elements for understanding the different mechanisms of action of bio-aerosols.

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MATRIX METALLOPROTEINASES (MMPs) IN FOLLICULAR FLUIDS (FF) AND FF-DERIVED EXTRACELLULAR VESICLES (EVs) FROM PATIENTS UNDERGOING TO *IN VITRO* FERTILIZATION (IVF) TECHNIQUES

F. Vaglica[1], M. Buttacavoli[1], F. Geraci[1], A. Ferro[2], C. Liuzzo[2], M. Galletta[2], S. Caudullo[3], P. Cancemi[1]

[1]STEBICEF Department, Viale delle Scienze Bld.16, Palermo; [2]PMA Center, Papardo Hospital, Messina, Italy; [3]Dipartimento Materno Infantile, Papardo Hospital, Messina, Italy
E-mail: patrizia.cancemi@unipa.it

Follicular fluid (FF) represent the optimal microenvironment for oocyte development, maturation and competence.[1] FF consists of plasma exudate containing hormones, metabolites and extracellular vesicles (EVs) necessary for the crosstalk between oocyte and somatic cells (cumulus cells, granulosa cells, and theca cells).[2] Moreover, it is known that extracellular matrix (ECM) remodelling is required in follicular development, ovulation, corpus luteum formation, and embryo implantation: a critical role in these processes belongs to matrix metalloproteinases (MMPs) and in particular the gelatinases MMP-2 and MMP-9.[3] Since the role of MMPs in human female infertility and in artificial ovarian hyperstimulation during the *in vitro* fertilization (IVF) cycles is not yet fully understood, we aimed to assess the activity levels of MMP-2 and MMP-9, in follicular fluid as well as in EVs isolated by ultracentrifugation from FF of 126 patients undergoing to IVF treatment. Our results revealed a consistent heterogeneity of MMPs levels between patients, with a higher gelatinolytic activity of proMMP-2 than proMMP-9. Interestingly, the active form of MMP-2 was detected only in FF-derived EVs. CRP levels (C-reactive protein), a specific marker of inflammation, was detected by Western blotting and correlated to MMPs expression. Clinical correlation with MMPs expression were also performed. In conclusion, MMPs-levels in FF and FF-derived EVs may be a reliable marker to predict success of IVF techniques. Considering their great potential, FF-derived EVs could be an interesting source for new prognostic markers. However, further studies are warranted to confirm these findings.

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rhNGF RESCUES HEARING IMPAIRMENT IN AGED SAMP8

M. d'Angelo, V. Castelli, A. Cimini

Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy
E-mail: michele.dangelo@univaq.it

Hearing loss is a common sensory disorder characterized by sensorineural hearing loss generally starting in individuals over 65 years, which impacts the quality of life and affects communication resulting in social isolation, and reduced well-being.[1,2] Aging triggers ROS generation in the inner ear with loss of sensory cells and hearing impairment. Hearing impairment is a relevant health problem due to numerous exogenous insults that induce inflammation and oxidative stress, leading to cell injury and hearing loss. Hearing impairment is the third most frequent sensory disorder in humans and there is no effective therapeutic approach to treat or counteract this disorder.[3,4] In this study, SAMP8 mice were selected as a model to detect the otoprotective effect of rhNGF and define if rhNGF could preserve the function of OHCs safeguarding hearing loss in aging mice. Specifically, electrophysiology studies reported a protective effect in animals treated with rhNGF both at the functional and morphological levels. Moreover, the histological characterization by SEM and immunohistochemistry of cochleae demonstrated the absence of stereocilia fusion of inner hair cells and an increase of the number of ribbon synapses and cochlear neurons after the compound treatment, corroborating electrophysiology, otoacoustic and functional data. Our results provide for the first-time evidence that the intranasal administration route efficiently targets the cochlea promoting the maintenance of IHC, OHC, and ribbon synapses and their functionality during aging, thus supporting the potential of NGF administration as a strong therapeutic approach to reduce the burden of ARHL also in humans. Our results provide the first demonstration that rhNGF, reaching the cochlea, exerted significative protective effects in preserving hair cells and number, thus indicating that intranasal administration is a minimal invasive and more efficient administration route.

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THE ROLE OF AUTOPHAGY IN GLIOBLASTOMA ONCOBIOLOGY

D. Gargano, M. Colardo, B. Colella, C. Lopa, G. Santoro, S. Di Bartolomeo

Department of Biosciences and Territory, University of Molise, Pesche (IS), Italy
E-mail: d.gargano@studenti.unimol.it

Autophagy is a highly conserved catabolic process which normally ensures cellular homeostasis degrading damaged organelles, protein aggregates and toxic debris. Under stressful conditions autophagy functions as pro-survival machinery by recycling unnecessary intracellular components to be used as an energy source for the adaptive response.[1] Therefore, abnormality in the autophagic flux is closely associated with human diseases, including cancers. However, the role of autophagy in tumor development and progression is extremely complex and context-dependent with a tumor suppressive and a tumor permissive function during the early and advanced stage of cancerogenesis, respectively.[2] Very poor is known about the role of autophagy in Glioblastoma (GBM), the most common and aggressive malignant primary brain tumor in humans. Autophagy modulates aggressiveness of GBM affecting resistance or sensitivity to therapy and also regulating invasion capabilities of tumor cells. In detail, autophagy induction downregulates EMT-like-Transition, induces beta-catenin relocalization and impairs EGFR-mediated pathway, resulting in a reduced aggressiveness.[3,4] Moreover, an elegant study performed in a Dropophila model has shown an autophagy suppression during gliomagenesis.[5] *Via* a transcriptomic data analysis, we recently highlighted a downregulation of the autophagy initiator ULK1 in all the four GBM subtypes, as well as in oligodendrogliomas and astrocytomas compared to normal brain. Western blotting analysis

from GBM patient biopsies unraveled a significant modulation in protein level of the main autophagy players in GBM samples in comparison with non-tumoral ones, supporting the hypothesis of an autophagy decreased proficiency within the tumoral mass. In order to identify the role of ULK1 and autophagy in GBM biology, genetic modulation of autophagy players will be performed and tumor aggressiveness evaluated in *in vitro* and, hopefully, in *in vivo* models.

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CHARACTERIZATION OF A ZEBRAFISH *SETD5* LOSS-OF-FUNCTION MODEL: INSIGHTS INTO NEURODEVELOPMENTAL AND BEHAVIOURAL DEFICITS ASSOCIATED WITH ASD

M. Digregorio[1], C. De Cesari[1], C. Gabellini[1], C. Pucci[1,2], D. Martini[1], R. Mosca[1], W. Norton[3], A. Zippo[4], V. Broccoli[5,6], A. Sessa[5], M. Andreazzoli[1]

[1]Department of Biology, University of Pisa, Italy; [2]Sant'Anna School of Advanced Studies, Pisa, Italy; [3]Department of Neuroscience, Psychology and Behaviour, University of Leicester, UK; [4]Laboratory of Chromatin Biology & Epigenetics, Center for Integrative Biology (CIBIO), University of Trento, Italy; [5]Stem Cell and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy; [6]CNR Institute of Neuroscience, Milan, Italy
E-mail: matteo.digregorio@phd.unipi.it

Loss-of-function (LoF) mutations in *SETD5* are strongly associated with intellectual disability (ID) and autistic spectrum disorders (ASD) in humans.[1,2] We have previously shown that *SETD5* is highly expressed in the brain, plays a critical role in neurodevelopment and its haploinsufficiency leads to reduced methylation of Histone 3 Lysine 36 (H3K36).[3] To better investigate the cellular and molecular mechanisms affected by *SETD5* haploinsufficiency, we generated a *setd5* LoF zebrafish model. We found that *setd5* is expressed in both maternal and zygotic transcripts in zebrafish and generated heterozygous fish carrying either wild-type or mutated maternal setd5 transcripts. Our study aimed to explore the effect of *setd5* LoF on cell cycle regulation and histological alterations in neural progenitor cell proliferation and neuronal differentiation. Our observations revealed that *setd5* LoF alters mRNA expression of cell cycle regulators, including *pcna* and *dyrk1a* homologs, which are associated with abnormal increases of neural proliferation in the adult brain. Furthermore, our data suggest that a potential dysregulation of neuronal differentiation/migration, occurring in the CNS of developing mutant embryos, could lead to abnormal brain development. These defects are coupled with a decreased expression of genes encoding synaptic and neurotransmission proteins such as *ddc, gad1a,* and *synaptophysin a*, which could affect neuronal transmission.[4] Interestingly, social behavioural tests indicate that the zebrafish setd5 mutants exhibit ASD-like features, making them a promising model for drug screening to ameliorate the behavioural phenotypes associated with *SETD5* mutations. Overall, this study highlights the crucial role of *SETD5* in neurodevelopment, provides insight into the underlying mechanisms of ID and ASD and paves the way to the identification of therapeutic treatments for individuals affected by *SETD5* haploinsufficiency.

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HIGH-FAT DIET INDUCES CELL DAMAGE IN RAT EPIDIDYMIS

D. Latino, S. Falvo, A. Santillo, G. Chieffi Baccari, M.M. Di Fiore

Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Caserta, Italy
E-mail: debora.latino@unicampania.it

In this study, we investigated the effects of five weeks high□fat diet (HFD: 21 carbohydrates, 29 proteins, 50 fat J/J; 19.85 kJ gross energy/g) on rat epididymis. Firstly, the results showed increased weight, food intake, and levels of serum cholesterol and triglycerides in rats of the HFD group compared with controls. Therefore, we studied the HFD-induced cellular response in the three regions of the rat epididymis (caput, corpus and cauda) by evaluating oxidative stress, apoptosis, proliferation, autophagy and selective autophagy. The results showed that HFD caused an increase of oxidative stress in all segments of the epididymis, indicated by increased levels of malondialdehyde (MDA) and decreased expression levels of superoxide dismutase 1 (SOD1). Increased apoptosis was recorded in the corpus and cauda segments, suggested by the increased expression levels of Bax, Cytocrome C and Caspase3, activated likely to eliminate dysfunctional cells. Further analyses suggested that HFD caused autophagy and lipophagy dysregulation in the epididymal corpus. More specifically, the expression levels of Perilipin3, a lipophagy marker, were increased in the corpus of HFD rats, whereas those of Beclin1 and ATG16, two autophagy markers, were decreased, indicating the inability of epididymal cells to counteract oxidative stress to maintain homeostasis. Finally, reduced expression levels of PCNA, a marker of cell proliferation, were observed in the corpus of HFD rats. Taken all together our findings suggest that HFD induces cellular damage prevalently in the corpus and cauda, regions playing a key role in monitoring epididymal health.

BLOOD TESTIS BARRIER PROTEIN IN AUTISM MODEL MOUSE

M. Prisco, S. Valiante, T. Chianese, T. Barra, G. Trinchese, M.P. Mollica, L. Rosati

Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: marina.prisco@unina.it

Autism spectrum disorders (ASD) are neurodevelopmental diseases with complex symptoms and whose neurobiological basis remains poorly understood. Pathogenesis seems to be linked to combination of genetic, autoimmune, environmental, and perhaps in utero risk factors leading to neuroinflammation.[1] A peculiar characteristic in human ASD is the presence of alterations at the level of the blood brain barrier and of intestinal epithelial barrier,

due to changes in tight junctions (TJ) protein expression:[2] inappropriate antigen trafficking through impaired barriers, followed by inflammation can be part of the chain of events leading to these disorders. To the best of our knowledge, there are no studies on the blood testis barrier. We are just starting to study the testis of BTBR T+tf/J mouse, a well-validated model of idiopathic autism: we here present the results of our investigations on testis structure and on expression of two protein of TJ, claudin-5 and connexin 43. In BTBR mouse the seminiferous tubules structure appears partially modified compared to the control and the TJ protein expression decreases. Treatment with a mix of dimethylglycine and B group vitamins, with anti-inflammatory action, seems to partially restore the testis condition.

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POTENTIAL COCKTAIL EFFECT OF NONYLPHENOL AND SEX HORMONES ON HUMAN PROSTATE CELLS

A. Mileo[1], F. Carrella[1], S. Boccia[1], R. Cominale[1], M. De Falco[1,2]

[1]Department of Biology, University of Naples Federico II, Naples; [2]National Institute of Biostructures and Biosystems (INBB), Rome, Italy
E-mail: madefalco@unina.it

Endocrine Disruptor Chemicals (EDCs) are a heterogeneous group of pollutants that have raised many concerns due to their chemical and physical features, such as high hydrophobicity and low water solubility.[1] Among these compounds, we can find Nonylphenol (NP) usually used in the manufacture of domestic, industrial, agricultural products and personal-care products, to improve their properties, as flexibility, durability, and transparency. This compound persists in different environmental matrices for a long time. So, they can bioaccumulate in adipose tissue and biomagnificate in food chain.[2] Human population can mainly be exposed to EDCs, through ingestion or skin contact. Thanks to estrogen like behaviour, NP can interfere with human endocrine system, through estrogen receptors (ERs) pathways.[3,4] Although NP has been extensively studied both *in vivo* and *in vitro*, it remains unclear if NP can interact with the sex hormone pathways, breaking the delicate sex hormonal balance. The aim of this study is to deepen the knowledge about the potential *cocktail effect* of NP with sex hormones on human non tumoral prostate cells (PNT1A). The first evidence has showed that all the mixtures induced cell proliferation through ERs pathways activation. NP additively interacted with testosterone (T) while in antagonistic manner with 17β estradiol (E_2). we showed that NP in mixture has competed with E_2 for the same ERα receptor site, delayed its cytoplasm nucleus translocation. Moreover, these mixtures could interfere with the dynamic structure of the cell cytoskeleton, modifying the average rate of the cell migration. Further studies will be carried out to investigate the cell inflammation status, which is the first step of carcinogenesis.

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METABOLIC REPROGRAMMING AND MODULATION OF MICROGLIA IN AMYOTROPHIC LATERAL SCLEROSIS

S. Vitolo[1], S. Migliarini[1], S. Scaricamazza[2], A. Votta[1], A. Ferri[2], M. Pasqualetti[1], E. Ferraro[1]

[1]Department of Biology, University of Pisa, Pisa; [2]Division of Experimental Neuroscience, IRCCS Fondazione Santa Lucia, Rome, Italy
E-mail: sara.vitolo@phd.unipi.it

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that results in the degeneration of upper and lower motor neurons. Motor neuron degeneration in ALS is associated with an inflammatory response that includes the activation of microglia.[1] The activation of microglia has been demonstrated in the spinal cord, whereas in the motor cortex it is still unclear.[2] To investigate the role of cerebral microglia in this pathology, we have analysed the morphology of microglia cells and the expression pattern of microglial molecular markers in the $SOD1^{G93A}$ mouse model of ALS, at two stages of the disease. This analysis has been performed on different CNS regions involved in the modulation of movement, *i.e.* cortex, striatum and cerebellum, and has been compared with the well-known features of spinal cord microglia. In addition, we have planned to evaluate the role of the metabolic modulator trimetazidine (TMZ) on microglia in ALS, since it had been shown that TMZ plays a protective role in the $SOD1^{G93A}$ mice.[3] Microglia can be divided into distinct subpopulations having different features among which different metabolic pathways. The anti-inflammatory subpopulations rely primarily on mitochondrial oxidative phosphorylation, whereas the pro-inflammatory subpopulations are more glycolytic.[4,5] We hypothesized that, by enhancing mitochondrial oxidative metabolism, TMZ might trigger a metabolic reprogramming on microglia which promotes an anti-inflammatory phenotype. In short, we expect to increase our knowledge on the specific contribution of microglia to the pathogenesis of ALS by a better understanding of the characteristics of microglia activation at the cerebral level, and to evaluate the effect of TMZ on microglia activation.

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EFFECTS OF TRIMETAZIDINE ON MITOCHONDRIAL DYSFUNCTION IN AMYOTROPHIC LATERAL SCLEROSIS SOD1^{G93A} CELL MODELS: AN ULTRASTRUCTURAL STUDY

M. Muzzi[1,2], S. Moreno[1,2], I. Salvatori[3], S. Scaricamazza[3], A. Spalloni[3], C. Valle[3], A. Ferri[3]

[1]Department of Science, University Roma Tre, Rome, Italy; [2]Lab. of Neurodevelopmental Biology, Neurogenetics and Molecular Neurobiology; [3]Lab. of Neurochemistry, IRCCS Fondazione S. Lucia, Rome, Italy
E-mail: maurizio.muzzi@uniroma3.it

Proper mitochondrial function is crucial for maintaining healthy neuronal cells, so that mitochondrial impairment contributes to the pathogenesis of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS).[1] This affects upper motor neurons (MNs) of the cerebral cortex and lower MNs of the spinal cord, leading to progressive weakening of voluntary muscles. Several ultrastructural investigations showed that, in both ALS patients and mouse models, MNs display mitochondria with morphological aberrations, associated with disturbed mitophagy, mitochondriogenesis and calcium homeostasis.[2] The metabolic modulator trimetazidine (TMZ) has recently been demonstrated to exert protective effects on *SOD1^{G93A}* mice, preventing spinal cord MNs loss and reducing neuroinflammation.[3] Even though the molecular mechanisms underlying such action are yet to be identified, TMZ effectiveness on *SOD1^{G93A}* mice is certainly linked to an improvement of mitochondrial functionality. In the present study we investigated the effects of TMZ on mitochondrial ultrastructure using primary cultures of cortical and spinal MNs obtained from *SOD1^{G93A}* mice and their wild type littermates. Focused Ion Beam/Scanning Electron Microscopy (FIB/SEM) analysis revealed the presence of dramatic mitochondrial alterations in both *SOD1^{G93A}* cell subtypes, as compared to their control counterparts. Disrupted inner membrane organization and fragmented cristae were frequently observed. Consistent with previous data, TMZ proved effective in significantly reducing mitochondrial abnormalities in both cortical and spinal *SOD1^{G93A}* cells. Moreover, regardless of cell type and genotype, TMZ treatment markedly increased the number of autophagosomes, suggesting that its beneficial effects on mitochondrial functionality may at least partially relate to its ability to promote mitophagy.

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MMP-2 AND MMP-9 EXPRESSION LEVELS IN CEREBROSPINAL FLUID AND DERIVED-EXTRACELLULAR VESISCLES FROM PATIENTS WITH MULTIPLE SCLEROSIS

F. Vaglica[1], M. Buttacavoli[1], E. Peri[1], C. D'Amico[1], S. Marino[1], F. Geraci[1], G. Salemi[2], P. Cancemi[1]

[1]STEBICEF Department, and [2]Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND), University of Palermo, Palermo, Italy
E-mail: fabiola.vaglica@unipa.it

In multiple sclerosis (MS), the most common immune-mediated neuroinflammatory disorder, demyelination and neuroaxonal damage depend on immunocompetent cells migration into the central nervous system (CNS), due to opening of the blood-brain barrier (BBB).[1] The BBB breakdown is mainly due to the activity of matrix metalloproteinases (MMPs), including gelatinases MMP-2 and -9, which play a central role in the immunopathogenesis of MS and therefore they have been previously proposed as candidate biomarkers for MS progression.[2,3] Gelatin zymography is a simple and reliable technique able to detect the enzymatic activity of both pro and active forms of MMP-2 and -9 enzymes, as well as complexes of proMMP-9 with their physiological inhibitors (TIMP-1) in different biological fluids.[4,5] In our study, gelatinase activity levels have been analysed in cerebrospinal fluid (CSF) samples and in Extracellular vesicles (EVs) isolated from CSF, as well as in sera of patients diagnosed with MS or other neurological diseases, considered as controls (NC). Our results revealed a different expression pattern of MMPs in CSF, CFS-derived EVs and serum. In particular, the active forms of MMP-2 and -9 were detected only in CSF samples and enriched in EVs, while the proMMP-9 dimers were revealed only in serum samples. ProMMP-9, proMMP-2 and proMMP-9/TIMP-1 were detected in both biological fluids. The expression levels of MMPs were also correlated with clinical phenotype. These evidences confirm that MMP-2 and -9 could be useful as potential biomarkers for monitoring MS disease and suggest that dysregulation of their proteolytic activity could be relevant in MS immunopathology. The possible enrichment of MMP-2 in EVs isolated by CSF deserves further investigation.

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INTERACTION BETWEEN β-ARRESTIN1 AND M2 MUSCARINIC RECEPTORS IN GLIOBLASTOMA CANCER STEM CELLS: IMPLICATION IN CELL PROLIFERATION AND SURVIVAL

C. Guerriero[1], G. Scanavino[1], I. Masi[2], G. Lupo[1], M. Matejcek[1], L. Rosanò[2], A.M. Tata[1]

[1]Department of Biology and Biotechnologies Charles Darwin, University of Rome Sapienza, Rome; [2] Institute of Pathology and Molecular Biology, CNR, Rome, Italy
E-mail: claudia.guerriero@uniroma.it

Glioblastoma multiforme is the most aggressive tumor in the central nervous system. It is characterized by the presence of an undifferentiated cell population named Glioblastoma Cancer Stem Cells (GSCs). Some embryonic genes can be reactivated in GSCs during malignant transformation, such as muscarinic receptors. Many reports suggest that muscarinic receptors can be involved in the regulation of the cell cycle, chemotaxis, and angiogenesis both in normal and tumor tissues. Recently we demonstrated that M2 receptor activation by orthosteric and allosteric agonists, inhibited cell proliferation[1,2] and counteracted cell survival involving PIP3K/AKT/mTOR pathway. β-Arrestins are ubiquitously proteins coupled with GPCRs. The best-known is β-arrestin1. It can regulate the GPCR desensitization and participate in the modulation of signal transduction pathways, cytoskeleton remodelling, cell proliferation, migration, and in the controlling of gene expres-

sion.[3] In this context, we started to investigate the possible interaction between β- arrestin1 and M2 receptor in GSCs and the possible modulation of β-arrestin1 modulation after M2 receptor activation. The first data obtained demonstrate that the M2 receptor stimulation caused downregulation of β-arrestin1 and its progressive translocation from nuclei to the cytoplasm. The interaction between M2 receptor and β-arrestin1 was evaluated in transfected GBM cell lines and GSCs, with two constructs overexpressing the two proteins conjugated with FLAG and GFP, respectively[4] and by GST pull down. These data obtained may contribute to a better understanding of the mechanisms downstream of M2 muscarinic receptors and the role of β-arrestin1 in this context.

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ROLES OF RETINOIC ACID SIGNALING IN NEURAL DIFFERENTIATION IN CEPHALOCHORDATES

M. Bozzo[1], D. Bellitto[1], E. Serafini[1], M. Pestarino[1], M. Schubert[2], S. Candiani[1]

[1]Department of Earth, Environmental ans Life Sciences, University of Genova, Genoa, Italy; [2]Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), Institut de la Mer de Villefranche, Sorbonne University, CNRS, Villefranche-sur-Mer, France
E-mail: matteo.bozzo@unige.it

The vertebrate neural tube has a dual origin: the anterior part forms by rolling up of the neural plate, which is induced from the ectoderm at gastrulation; the posterior part, conversely, derives from axial progenitors (APs) that emerge at the end of gastrulation and are located in the tail bud,[1] the remnant of the blastopore lips. The cephalochordates (amphioxus) are the earliest-diverging chordate group, making them the most informative for defining ancestral chordate conditions. Like in vertebrates, the amphioxus anterior neural tube derives from the neural plate, while the tail bud produces the posterior neural tube, notochord, and somites during subsequent development.[2,3] However, while the neural progenitors of vertebrates rapidly respond to intercellular signalling and differentiate into specific neural types, in amphioxus the differentiation of posterior neural progenitors is delayed. Consequently, in tail-bud-stage amphioxus embryos, while the anterior neural tube contains differentiated neurons and glial cells, the posterior third of the larval CNS does not show hallmarks of neural differentiation.[4] Here, by pharmacological manipulations and *in situ* expression analysis of selected marker genes, we show that, in amphioxus, retinoic acid (RA) signalling has different roles in neural differentiation depending on the anteroposterior position of the responding precursors. In the anterior part of the developing neural tube, three regions can be distinguished based on their responsiveness to RA.[5] Furthermore, we provide evidence that RA signalling is also implicated in the differentiation of the tail bud-derived precursors of the posterior neural tube.

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ZEBRAFISH DNM1A GENE PLAYS A ROLE IN THE FORMATION OF AXONS AND SYNAPSES IN THE NERVOUS TISSUE

C. Bragato[1], A. Pistocchi[2], G. Bellipanni[3], S. Confalonieri[4], J. Balciuniene[3], F.M. Monastra[2], S. Carra[5], G. Vitale[2,5], P. Mantecca[1], F. Cotelli[6], G. Gaudenzi[5]

[1]Department of Earth and Environmental Sciences, POLARIS Research Center, University of Milano-Bicocca, Milan, Italy; [2]Department of Biotechnologies, University of Milan, Milan, Italy; [3]Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, USA; [4]IEO, European Institute of Oncology, Milan, Italy; [5]Lab. of Endocrine and Metabolic Research, Istituto Auxologico Italiano, Milan, Italy; [6]Department of Biosciences, University of Milan, Milan, Italy
E-mail: cinzia.bragato@unimib.it

Clathrin-mediated endocytosis (CME) is the most important endocytic way for the internalization of cargoes from the plasma membrane of eukaryotic cells into the cytoplasm,[1] and plays an important role in nerve terminals, where is involved in the recycling of synaptic vesicles after the release of neurotransmitters.[2] Several proteins are engaged in this process, such as the classical dynamins (DNMs). DNMs assemble into helical polymers at the necks of clathrin-coated endocytic pits and mediate fission of the underlying tubular membrane to generate a free endocytic vesicle. Three DNM genes are present in mammals. DNM1 is expressed at high levels in neurons, where it takes place in the recycling of synaptic vesicles; DNM2 is ubiquitously expressed, while DNM3 is found in the brain and in the testis.[3] In the teleost zebrafish (*Danio rerio*), two orthologs of the human DNM1 are present, named *dnm1a* and *dnm1b*. In particular, we focused on the role of *dnm1a* during embryonic development through the morpholino-based gene knockdown technology, characterizing this gene for the first time. Our data demonstrate that *dnm1a* has a nervous tissue-specific expression pattern and plays a role in the formation of both axon and neuromuscular junctions.

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MULTIPLE APPROACHES REVEAL ALTERED LIPID METABOLISM, NEUROTRANSMITTER RELEASE AND NERVOUS CONDUCTION IN A ZEBRAFISH MODEL OF ALEXANDER DISEASE

D. Bellitto[1], M. Bozzo[1], S. Ravera[2], N. Bertola[2], E. Fedele[3], M. Balbi[3], M. Milanese[3], L. Pastorino[4], D. Di Lisa[4], F. Rosamilia[5], P. Barboro[6], G. Coronel Vargas[6], E. Iervasi[6], M. Ponassi[6], A. Profumo[6], P. Romano[6], C. Rosano[6], S. Candiani[1§], T. Bachetti[6]

[1]Department of Earth, Environment and Life Sciences, University of Genoa; [2]Experimental Medicine Department, University of Genoa; [3]Department of Pharmacy, University of Genoa; [4]Department of Informatics, Bioengineering, Robotics, and Systems Engineering, University of Genoa; [5]Health Science Department, Biostatistic Unit, University of Genoa; [6]IRCCS Ospedale Policlinico San Martino, Genoa, Italy
E-mail: deianira.bellitto@edu.unige.it

Alexander Disease (AxD) is a rare pathology affecting astrocytes, caused by mutations in the GFAP gene encoding for the GFAP protein, an intermediate filament of astrocytes.[1] These mutations cause the formation of protein aggregations known as Rosenthal Fibers that led to neurodegeneration. In this work, by using the Tol2 system, we created an AxD model expressing the human GFAP p.R239C mutation (p.hR239C-GFP) in glial cells, under the control of zebrafish gfap promoter.[2] AxD mutant line, on which we performed transcriptomic and proteomic analysis, shows GFAP aggregates, well resembling the hallmark of the disease. Our preliminary data revealed increased oxidative stress and lipid peroxidation in p.R239C mutants. In line with the increased lipid peroxidation, the GFAP mutant line shows increased lipid synthesis and decreased β-oxidation. The analysis of oxidative metabolism highlights that the mutant line has a dramatic decrease in ATP synthesis and oxygen consumption. Moreover, to further investigate nervous conduction and neurotransmitter release, we performed the first zebrafish synaptosomal preparation and MEA analysis on our mutant model, revealing a decrease in glutamate release and a perturbation in the electric activity. Together, these results confirm the reliability of our zebrafish model and open to further investigation into the role of lipid metabolism and glutamate transport in AxD.

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PROTECTIVE EFFECTS OF MELATONIN IN REDUCING OXIDATIVE STRESS IN A MODEL OF AGING NEUROGLIA: FOCUS ON THE KIR2.1 CHANNEL ACTIVITY

A. Remigante[1,2], S. Spinelli[1,2], A. Marino[1], M. Pusch[3], S. Dossena[2], R. Morabito[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy; [2]Institute of Pharmacology and Toxicology, Paracelsus Medical University, Salzburg, Austria; [3]Biophysics Institute, National Research Council, Genoa, Italy
E-mail: aremigante@unime.it

Epilepsy is a brain disease and its prevalence increases with age. In this context, aging-related oxidative stress (OS) could be regarded to as a possible mechanism involved in epileptogenesis. The neuronal glia plays a crucial role in epilepsy by controlling neuronal hyperexcitability.[1,2] Among experimental OS-related aging models, D-Galactose (D-Gal) long-term exposure is considered the most similar to natural aging.[3] Here, we explored melatonin (Mel) effect, a free radical scavenger, in a D-Gal-induced aging model in glioblastoma U87-MG cells. Screening of all fifteen Kir isoforms revealed that the predominant transcript in U87-MG cells corresponds to Kir2.1. D-Gal exposure did not have an obvious cytotoxicity but resulted in an increased production of ROS and lipoperoxidation levels, decreased amount of membrane protein -SH groups, as well as stimulation of the activity of CAT and SOD. Interestingly, D-Gal treatment was associated with a decrease of the inwardly rectifying K+ currents sensitive to ML-133 (specific inhibitor of Kir2.1 channels). Mel pre-treatment prevented D-Gal-induced OS damage, as well as the decrease in the endogenous Kir2.1 current. This latter finding was also confirmed following Kir2.1 ectopic expression in NIH/3T3 cells. Our findings show i) a novel Kir2.1 channel modulation that likely occurs in OS conditions; ii) a crucial role of Mel in alleviating OS-induced damage, including the suppression of Kir2.1 ion current. We suggest that the inhibition of Kir2.1 channel in glia cells could alter extracellular K+ buffering and contribute to neuronal hyperexcitability associated with OS. Thus, we propose Mel as an excellent candidate to counteract oxidative alterations in epileptogenesis during aging.

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UNVEILING THE HIDDEN ROLE OF NATURAL KILLER CELL TRIGGERING RECEPTOR (NKTR) GENE IN NEURAL CREST CELLS (NCCs) DEVELOPMENT

M. Orefice[1,2*], R. Oleari[2*], D. Corsinovi[1], F. Amoruso[2], A. Cariboni[2], M. Ori[1]

[1]Unit of Cellular and Developmental Biology, Department of Biology, University of Pisa, Pisa; [2]Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy
E-mail: michela.ori@unipi.it; anna.cariboni@unimi.it
** These authors contributed equally*

By examining exome sequencing data, we identified two patients with a rare genetic neurodevelopmental syndrome characterized by developmental delay, dysmorphic facial features and carrying a homozygous nonsense mutation in the *NKTR* gene. This variant (c.2260C > T - p.R754*) is located at a highly conserved residue in the SR-rich domain (serine/arginine) of the NKTR protein and is likely pathogenic. *NKTR* encodes for a poorly studied protein that was initially identified as a membrane co-receptor for target recognition of natural killer cells.[1,2] However, due to the lack of cell and animal models, the role of *NKTR* during embryonic development is still unclear. In our study, we firstly demonstrated that mouse and zebrafish *NKTR* orthologs are expressed in Neural Crest Cell (NCC)-derived structures during embryonic development. Moreover, *Nktr* expression overlaps with Alcian blue staining and Sox9 expression in chondrogenic structures of the developing mouse head, suggesting an involvement of Nktr in craniofacial development. Next, we used Morpholino phosphorodiamidate-antisense oligonucleotides (MPO) to knockdown *nktr* expression in zebrafish embryos, which exhibited defects in head cartilage formation and an aberrant expression pattern of the NCC markers *dlx2* and *crestin* when compared to control. Finally, by knocking down nktr expression through MPOs in a transgenic reporter zebrafish line, known as Tg(*sox10::GFP*), we observed alterations in neural crest formation and NCC migration, suggesting that the craniofacial defects exhibited by *nktr* morphants may depend on defective NCC development. To corroborate and validate our findings, we designed a CRISPR/Cas13d-based knockdown strategy in zebrafish. Further analyses will be performed *in vitro* and *in vivo*, in both mouse and zebrafish, to expand our knowledge of the specific contribution of NKTR to the pathogenesis of craniofacial defects observed in both human patients and zebrafish *nktr* morphants, as well as its possible involvement in neurocristopathies. We believe that our study will provide insights and results that may accelerate the identification of novel therapeutic interventions for developmental disorders associated to defective NKTR activity and provide a proof-of-principle for an integrated pipeline using zebrafish and mouse models that efficiently uncovers the mechanisms underlying other rare genetic diseases.

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FUNCTIONAL AND NEUROANATOMICAL DISCONNECTIVITY IN ASD MOUSE MODELS

N. Barsotti[1], M. Pagani[2,3], A. Bertero[1,2,4], A. Gozzi[2], M. Pasqualetti[1,2]

[1]Department of Biology, University of Pisa, Pisa, Italy; [2]Functional Neuroimaging Laboratory, Istituto Italiano di Tecnologia, Center for Neuroscience and Cognitive Systems, University of Trento, Trento, Italy; [3]Autism Center, Child Mind Institute, Inc., New York, NY, USA; [4]Department of Biology, Neurosciences Institute, University of Texas at San Antonio, San Antonio, Texas, USA

E-mail: noemi.barsotti@unipi.it

Autism spectrum disorder (ASD) are a heterogenous group of neurodevelopmental disorders characterized by altered social interactions, communication difficulties and repetitive patterns of behaviour.[1] Such symptoms have been associated with alterations of functional connectivity in brain regions involved in emotional regulation and high-order association functions.[2] However, the neurophysiological underpinnings of these connectional derangements are largely unknown. Here we present the results of studies conducted on three different monogenic ASD mouse model (*i.e.,* Shank3, CNTNAP2 and TSC2 murine lines), in which loss-of-function mutation of a single gene recapitulates the human pathologies.[3,4,5] Through fMRI technique we investigated functional connectivity, describing aberrant activity, either reduction or increase, involving different brain regions. Viral-based neuroanatomical analyses revealed neuronal connectivity defects, providing structural evidence to the functional dysregulation. Strikingly, in TSC2 murine model, a pharmacological treatment allowed normalization of functional connectivity through reduction of dendritic spine density to control level, which were translated into the rescue of the behavioral phenotype.[5] In these studies, we exploited monogenic ASD mouse model to investigate brain connectivity in terms of functional activity and neuronal circuitries, linking the deficits in neuronal activity to neuroanatomical defects, which, in some cases, can be corrected to rescue both functionality and behavioral pattern.

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INHIBITION OF BET PROTEINS BY JQ1 PREVENTS ROTENONE-INDUCED OXIDATIVE STRESS IN A CELL MODEL OF PARKINSON'S DISEASE

D. Pensabene[1,2,3], N. Martella[1], S. Moreno[2,3], M. Segatto[1]

[1]Department of Biosciences and Territory, University of Molise, Pesche (IS); [2]Department of Science, University Roma Tre, Rome; [3]Laboratory of Neurodevelopment, Neurogenetics and Neuromolecular Biology, IRCCS Santa Lucia Foundation, Rome, Italy

E-mail: dan.pensabene@stud.uniroma3.it

Oxidative stress is a key pathological feature in patients affected by Parkinson's Disease (PD). Indeed, along with protein aggregation and neuroinflammation, dysmetabolism of reactive oxygen species (ROS) is considered one of the leading causes of progressive dopaminergic cell death in the *Substantia nigra pars compacta* (SNpc), resulting in motor and non-motor symptoms.[1] Recent evidence suggests that the modulation of a class of proteins, referred to as Bromodomain and Extra-Terminal (BET) proteins, plays a crucial role in maintaining neuronal homeostasis. BET proteins control gene expression through epigenetic regulation and are involved in the onset and progression of several neurological disorders.[2] In this regard, the impact of BET modulation on PD is still elusive. Thus, we aimed at evaluating whether the BET inhibitor JQ1 could ameliorate the parkinsonian phenotype in a rotenone-induced cellular model of PD. By restoring ROS metabolism through the activation of different molecular pathways, BET protein inhibition mitigates rotenone-induced ROS overproduction, α-synuclein aggregation and mitochondrial dysfunction, resulting in an overall suppression of death in SH-SY5Y cell model evaluated through TUNEL assay and other biochemical analysis. In conclusion, our data highlight a novel role for BET proteins in neurodegeneration, suggesting that their inhibition may represent a promising approach to counteract PD neuropathology.

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BET INHIBITION ATTENUATES CHOLESTEROL METABOLISM DERANGEMENTS IN RETT SYNDROME

N. Martella, M. Varone, M. Colardo, M. Segatto

Department of Biosciences and Territory, University of Molise, Pesche (IS), Italy

E-mail: n.martella@studenti.unimol.it

Rett syndrome (RTT, OMIM ID: 312750) is a neurodevelopmental disorder characterized by loss of acquired cognitive, social, and motor skills.[1,2] In most cases, RTT is caused by mutations in the MECP2 gene, which codifies for a transcriptional repressor. Different studies have revealed extensive metabolic impairments as hallmark of RTT, particularly alterations in cholesterol homeostasis are observed in both preclinical mouse models and patients.[3,4] Since BET proteins have emerged as epigenetic regulators strongly involved in the regulation of cholesterol homeostasis, the aim of this work is to evaluate whether BET inhibition could counteract the changes in cholesterol metabolism observed in RTT phenotype. To reach this objective, we used primary fibroblasts derived from both healthy and RTT individuals as experimental models. Filipin staining and enzymatic colorimetric tests showed

significant excess of cholesterol levels in RTT cells. Coherently, Western blot and immunofluorescence analysis confirmed alterations in several proteins and enzymes controlling cholesterol metabolism. Interestingly, JQ1 treatment significantly attenuated these abnormalities in RTT fibroblasts, leading to the restoration of intracellular cholesterol content. Taken together, these data suggest that BET inhibition may represent an effective pharmacological approach to restore the defects in cholesterol metabolism observed in the RTT phenotype.

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NSC-34 MN-LIKE CELLS TRANSFECTED WITH MUTANT SOD1 RECRUIT RAW 264.7 MACROPHAGES: THE START OF A FATAL COMMUNICATION

E. Carata*, M. Muci*, S. Di Giulio, S. Mariano, E. Panzarini

Department of Biological Sciences and Technologies, University of Salento, Lecce, Italy
E-mail: elisabetta.carata@unisalento.it
** These authors contributed equally*

The EVs/macrophages/neurons axis could represent a new hope in therapeutic strategy for amyotrophic lateral sclerosis (ALS), as many studies have shown that macrophages are critical to the progression of inflammation with a particular role in the regulation of the degree of inflammation. In addition, the role of EVs in recruiting macrophages into CNS or in mediating inflammatory status is becoming a very attractive field of research and EVs cargo offers an interesting and relatively untrapped area of therapeutic target discovery. EVs play a role in the disease pathogenesis through the transfer and subsequent intracellular accumulation in target cells of both pathological proteins and inflammatory mediators able to modulate the activation/damage of nervous cells. Despite this, relatively little is known about these mechanisms and how they are altered in neuroinflammation. In the current study, we cultured Raw 264.7 macrophages on the upper chamber of the 8 mm pore-size PET transwell and mSOD1 (G93A, A4V, G85R, G37R) NSC-34 cells in the lower chamber for 12, 24 and 48h to evaluate the recruitment of macrophages and the response of both macrophages and motor neurons. Macrophages are actively recruited towards mSOD1 NSC34 at 24h of incubation; the extent of macrophages recruitment depend on the mutated cells type present in the lower chamber (A4V>G93A>G85R>G37R). The analysis of mRNA levels of several cytokines suggested an early pro-inflammatory response of Raw 264.7 macrophages, that, in turn, increased the gene expression of pro-inflammatory cytokines also in NSC-34 cells. Moreover, caspase-1 and caspase-3 were sequentially activated in motor neurons, suggesting an exacerbation of cell damage. Finally, the analysis of the cargo of EVs released by mSOD1 NSC-34 revealed the transport of pro-inflammatory mediators that can be involved in macrophages activation and recruitment. Overall, the results of the present research highlight that macrophages have a high potency as target to modulate ALS disease. However, further studies are needed to explore the molecular mechanisms through macrophages activity impact ALS progression and the role of EVs cargo in exacerbating inflammation.

TEMPERATURE VARIATION INDUCES NEUROTOXICITY IN *DANIO RERIO*

F. Grassi Scalvini[1], S. Nonnis[1,2], E. Maffioli[1], G. Tedeschi[1,2], M. Fazzina[3], F. Frabetti[4], C. Cioni[5], M. Toni[5]

[1]Department of Veterinary Medicine and Animal Science, University of Milan, Milan; [2]CRC "Innovation for Well-being and Environment" (I-WE), University of Milan, Milan; [3]Department for Life Quality Studies - QUVI, University of Bologna, Rimini; [4]Department of Medical and Surgical Science, University of Bologna; [5]Department of Biology and Biotechnologies "Charles Darwin", Sapienza University, Rome, Italy
E-mail: mattia.toni@uniroma1.it

Previous studies from our research group suggest that in Danio rerio environmental temperature variation heavily alters locomotor activity but also complex behaviours such as anxiety, social behaviour, aggression, learning, and cognitive behaviours.[1,4] Overall, our results suggest that the molecular and behavioural effect generated by temperature variation can be framed in the context of neurotoxicity. Exploiting all the previously obtained proteomic data, we now focus on temperature variation-generated neurotoxicity to fully understand its molecular mechanism, to identify the most important pathways altered by temperature variation and to recover key drivers of pathogenesis. From our previous works, we extrapolated all the data listing the proteins differentially expressed (either increased, decreased or exclusively expressed in one single conditions) in the comparisons: 18°C *vs* 26°C (kept as a control) and 34°C *vs* 26°C upon acute[3] and chronic exposure[4], $BDNF^{+/-}$ (HT) *vs* wild type (WT) and $BDNF^{-/-}$ (KO) *vs* WT at 26°C and 34°C.[2] A total of eight datasets were obtained and a tox analysis was conducted by IPA setting a significance level of $p \leq 0.05$. Considering the top five most enriched terms possibly related to neurotoxicity, in terms of pathways, networks and genes, two major classes emerge from the toxicological analysis and are present in all data sets: neurological diseases and nervous system development. In particular, most of the proteins whose expression is altered by thermal stress are related to synapsis and cell projections.

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DNA DAMAGE IN THE BRAIN OF ZEBRAFISH EXPOSED TO ALUMINUM

I. Ferrandino, A.R. Bianchi, A. La Pietra, T. Capriello, V. Guerretti, D. Lucariello, A. De Maio

Department of Biology, University of Naples Federico II, Naples, Italy
E-mail: ida.ferrandino@unina.it

Aluminum (Al) is a widespread metal in the environment but with no role in metabolic processes. It is unsafe for human health and its accumulation in the brain has been associated with neurodegenerative diseases.[1] In previous studies, we demonstrated that Al increases apoptosis, oxidative stress, and genotoxicity in zebrafish embryos.[2] In adults, this metal alters their behavior and induces changes in the histology and the oxidative state of the brain.[3]

Moreover, we also demonstrated that poly(ADP-ribosyl)ation activation occurs after 10 and 15 days of Al exposure.[4] Poly(ADP-ribosyl)ation is a covalent and reversible post-translational modification of proteins, catalyzed by a family of enzymes, the poly(ADPR) polymerases (PARPs). Nuclear PARP1 and PARP2 are considered "sensors" of DNA damage and their activation is involved in both physiological processes (DNA repair and maintenance) and in various pathologies.[5,6] In the present study we have characterized, for the first time, the complete poly(ADP-ribosyl)ation system in the brain of adult zebrafish exposed to 11 mg/L Al for 10, 15, and 20 days. The results showed the existence of several PARP isoforms, among which the counterpart of human PARP1. Two PARG isoforms with a molecular weight of about 68 kDa and 87 kDa respectively were identified too. The highest levels of PARP activity and PAR synthesis were detected at 10 and 15 days of treatment. On the contrary, they drastically reduced after 20 days. We suppose that exposure to Al for 10 and 15 days produces DNA damage with consequent activation of PARPs. At these times, PARG activation is necessary to avoid the accumulation of PAR, which inhibits PARP and promotes parthanatos. At longer times (20 days), instead, the reduction of PARP activity and PAR synthesis could represent a mechanism of the neuronal cells to avoid intracellular energy consumption and ensure cell survival.

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DISCOVERING NEW lncRNAs IN ZEBRAFISH: CHARACTERIZATION OF *LOC100535512* IN THE DEVELOPING AND ADULT CENTRAL NERVOUS SYSTEM

M. Fazzina[1], L. Cogliandro[2], M. Bergonzoni[3], R. Casadei[1], F. Frabetti[2]

[1]Department for Life Quality Studies - QUVI, University of Bologna, Rimini; [2]Department of Medical and Surgical Sciences - DIMEC, University of Bologna, Bologna; [3]Department of Biomedical and Neuromotor Sciences - DIBINEM, University of Bologna, Bologna, Italy
E-mail: martina.fazzina2@unibo.it

Long non-coding RNA (lncRNAs) are non-coding transcripts longer than 200 nucleotides that have been shown to play a key role in a wide range of physiological and pathological processes at the transcriptional, post-transcriptional and epigenetic levels.[1] The study of lncRNAs in Zebrafish (*D. rerio*) is challenging due to the lack of evolutionary conservation of their sequences.[2] Nonetheless, potential orthologs lncRNAs can be located in syntenic loci, with identical neighboring genes, in different species.[3] We identified a genomic region on Zebrafish's chromosome 17 containing a putative lncRNA (*LOC100535512*) that is syntenic to a trait in human chromosome 14 where the lncRNA LINC00520, which we discovered may be associated with Parkinson's disease (PD),[4] is located. Our primary goal is to evaluate gene expression and modulation of *LOC100535512* in the Zebrafish model during development and in adult tissues. To this end, we first sequenced the *LOC100535512* transcript variant 1 (predicted), an approach essential for downstream applications. Then, we observed a significant increase in the lncRNA expression levels in Zebrafish embryos during early developmental stages, specifically at the onset of epibolic movements and during the gastrulation period, when primary neurogenesis occurs. Moreover, the analysis of *LOC100535512* in adult animals revealed higher gene expression in the brain compared to other organs or tissues. Additionally, to assess the orthology with the human lncRNA and to evaluate the hypothetical regulatory action of *LOC100535512* on the antioxidant response *in vivo*, we are carrying out Rotenone treatments on Zebrafish larvae, which represents an established PD model.[5] Our findings suggest an essential role for *LOC100535512* during Zebrafish development, particularly in the stages in which the neural plate, representing a "primordial brain", is formed. Notably, the lncRNA gene expression remains high in the adult Zebrafish brain, indicating a possible functional role for it in the regulatory circuits of the nervous system. This study will be useful to understand the physiological role of an uncharacterized lncRNA in Zebrafish embryology and to confirm its suggested orthology with a human lncRNA in order to investigate its function, specifically in the central nervous system.

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EXPLOITING ZEBRAFISH TOOLS TO EVALUATE BIOINTERACTIONS AND ADVERSE EFFECTS OF SILICA- AND COPPER-BASED NANOMATERIALS

C. D'Abramo, B. Negrini, R. Mazzotta, P. Floris, P. Bonfanti, A. Colombo, C. Bragato, P. Mantecca

[1]Department of Earth and Environmental Sciences, POLARIS Research Center, University of Milano-Bicocca, Milan, Italy
E-mail: c.dabramo@campus.unimib.it

To guarantee a safe and sustainable development of nanotechnologies, the human and environmental (nano)toxicological outcomes of new nanomaterials (NMs) should be evaluated since the design phase, with special attention to the relationship between the NM physico-chemical (P-chem) properties and the biological responses. In this work, zebrafish (*D. rerio*) development is proposed as a valid model to screen and compare the biological effects of SiO_2- and CuO-based nanoparticles (NPs), differing for the synthesis process and surface functionalization. Commercial SiO_2, ZnO@SiO_2 hybrid NPs and bio-nanosilica from rice husk (SiO_2- RHSK) NPs, as innovative fillers for tyre rubber and polyurethane foams, and CuO and Zn-doped CuO NPs, as effective antimicrobial agents, have been assessed by Fish Embryo acute Toxicity (FET) test, in parallel with their P-chem characterization. Both commercial and bio-SiO2 NPs had very mild embryotoxic effects, while their surface modification with ZnO enhanced the lethality, malformation rate and delayed the embryos hatching. The CuO NPs induced no lethality and only moderate malformation effects, but severely interfered with the embryos hatching. The Zn doping is able to modulate the CuO NP-induced adverse outcomes by lowering the effect on the embryos hatching, but slightly increasing lethality. Zebrafish developmental features, like malformation and especially hatching rate are here demonstrated to be predictive tools to assess NMs adverse effects related to P-chem characteristics.

IDENTIFICATION OF A FUNCTIONAL PREDICTIVE ASSAY TO TEST DEVELOPMENTAL NEUROTOXICITY IN THE *XENOPUS LAEVIS* TADPOLE

F. Di Renzo[1], M. Battistoni[1], R. Bacchetta[1], F. Metruccio[2], E. Menegola[1]

[1]Department of Environmental Science and Policy Università degli Studi di Milano; [2]ICPS, ASST Fatebenefratelli Sacco, Milan, Italy
E-mail: francesca.direnzo@unimi.it

In compliance to animal welfare 3Rs principle there is a great demand for refined tests alternative to classical mammal teratogenicity tests. We propose, using the refined alternative amphibian method (R-FETAX), a neurobehavioral test adapted to evaluate *Xenopus laevis* tadpole swimming profile. We exposed *X. laevis* embryos during neurobehavioural development (NF stages 37-46 corresponding to the spontaneous swimming acquisition period) to molecules involved in developmental neurotoxicity. In particular we tested the antiepileptic drug VPA (500-1500 mM) and two bisphenol-plasticizers (BPA 10-25 mM and BPB 5-10 mM). Neurobehavioral deficits were described applying a tracking application specifically designed to support animal behavioral analyses (AnimalTracking) to test *X. laevis* swimming capabilities. The AnimalTracker consists of three main modules: the Tracker module (responsible for image processing), the Zone Designer module (providing tools to create custom-made investigation areas), the Tracking Analyzer module (to define and obtain parameters). The motor behaviour of the tadpoles was evaluated on tadpoles were transferred into a 27 mm plastic cylinder, representing the arena filled with FETAX solution on an under-illuminated stereomicroscope. Larvae were allowed to acclimate for 60 s and videos taken for 60 s using a digital camera and subsequently analysed using the AnimalTracker plugin and the free image processing program ImageJ. On the basis of free swimming evaluated in controls, we set the region normally not invaded by swim as "inner circles area" (0.75 internal diameter considering 1 the arena diameter). Selected parameters were: total/ outer ring/ inner circle distances, mobility time in the different sectors, time spent in the different sectors, speed. The tested molecules induced neurobehavioural deficits in a concentration-related manner: VPA reduced total distance and speed, BPA and BPB increased the inner circle distance altering the swimming routes. We suggest the amphibian swimming test as a valid additional test for the evaluation of chemicals suspected to alter the neural development.

CELL PROFILING OF THE EMBRYONIC INTEGUMENT OF THE MODEL FROG *PELOPHYLAX* KL. *ESCULENTUS* BY HISTOCHEMICAL AND ULTRASTRUCTURAL METHODS

G. Scillitani, D. Semeraro, M.V. Guglielmi, D. Mentino, M. Mastrodonato

[1]Department of Bioscience, Biotechnology and Environment, University of Bari, Bari, Italy
E-mail: giovanni.scillitani@uniba.it

Current research requires effective experimental models to assess how emerging xenobiotics affect biological systems. Amphibians such as *Xenopus laevis* have been widely used, but it is necessary to use native species as bioindicators and biomarkers for monitoring local environmental pollution. In our laboratory, we are evaluating the use of the embryonic stages of the common water frog, *Pelophylax* kl. *esculentus* by analysing its epidermis, a system interface with the environment. Although anurans such as *X. laevis* and the common water frog are quite distant phylogenetically, *P.* kl. *esculentus* being autochthonous, allows to monitor local environmental conditions. Embryos epidermis at (Gosner 21) consists of a bilayered epithelium with a basal and an apical layer. Five cell types were evidenced: basal cells (BC), ciliated cells (CC), goblet cells (GC), small secretory cells (SSC), and ionocytes (IC). BCs constitute the basal layer of the epidermis. They have a large nucleus and are involved in proliferation and differentiation of the other cell types. CC present several cilia on the apical surface. They show an alcianophilic sub-apical portion. TEM analysis reveals small vesicles just below the surface, central nucleus, and evident yolk plaques. GC are the most abundant in the epithelium and are characterized by apical vesicles secreting a metachromatic, PAS-positive, alcianophilic mucus. With TEM, these cells appear low electron-dense, with a large central nucleus. SSC are cup-shaped with several apical vacuoles and are often associated with CC and IC. Vacuoles content appears PAS-positive an alcianophilic. IC are highly electron dense under TEM, with numerous apical microvilli and with vacuoles filled with granular material. FITC-WGA lectin binding indicates glycosaminylated and/or sialylated residuals in apical position of CC, GC, and SSC. These cell types are probably involved in secreting and regulatory activities. The described features appear to change in treatments with some xenobiotics, such as engineered metallic nanoparticles, thus supporting the embryos of *P.* kl. *esculentus* use, as model in ecotoxicological monitoring.

IDENTIFICATION OF A NEW DEVELOPMENTAL SCORING SYSTEM APPLICABLE TO FETAX (FROG EMBRYO TERATOGENICITY ASSAY: *XENOPUS*)

M. Battistoni[1], F. Di Renzo[1], F. Metruccio[2], E. Menegola[1]

[1]Department of Environmental Science and Policy Università degli Studi di Milano; [2]ICPS, ASST Fatebenefratelli Sacco, Milan, Italy
E-mail: maria.battistoni@unimi.it

The quantitative estimation of embryonic growth and development is a major concern in developmental toxicity evaluation. Exposure to xenobiotics or pathological conditions in pregnancy may result in embryo-/foetal lethality, gross malformations and developmental degree variations (delays or overgrowth defined also in humans as young/old-for-age and small/large-for age). A precise estimation of overall embryonic development and the evaluation of treatment-related deviation from the normal developmental grow rate is possible by application of quantitative morphological methods (scoring systems). The most accurate score for embryotoxicity evaluation is the Brown and Fabro scoring system designed for rat embryos cultured *in vitro*. We used this scoring system for post-implantation rat embryo exposed *in vitro* to xenobiotics for decades and this allowed us to obtain significant indication on substance- and dose-related developmental impairments. The aim of the present work is to describe a quantitative assessment of the development of *Xenopus laevis* embryos applicable to FETAX (Frog Embryo Teratogenesis Assay: *Xenopus*) methodology. Seven morphological features observed macroscopically in tadpoles at Nieuwkoop an Faber stages 40-47 were selected as representative of developmental degrees. Up to eight developmental stages of each feature were defined and assigned scores of 0 to 7; the numerical total of scores for each individual embryo was taken as the overall morphological score (total score). This scoring system was set observing the normal development of 148 unexposed

embryos maintained in our controlled experimental conditions (R-FETAX). To test the applicability of this new scoring system in developmental toxicity studies, we evaluated samples exposed to different teratogens at concentrations not inducing delay in intestine coiling (a parameter usually recognised as indicator of developmental degree): ethanol (17-85 mM), triadimefon (15.625-62.5 mM), β-carotene (1.4-5.6 mM). Data were modelled by PROAST software and dose-response curves derived. Results support the use of the present scoring system to quantitatively assess *X. laevis* development variations in embryotoxicity studies.

EVALUATION OF THE REPRODUCTIVE BIOLOGY OF THE EUROPEAN SARDINE IN THE ADRIATIC SEA

G. Chemello[1], G. Cerrone[1], V. Tavolazzi[1], F. Donato[2], F. Tiralongo[3], O. Carnevali[1], G. Gioacchini[1]

[1]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona; [2]Institute for Biological Resources and Marine Biotechnologies (IRBIM), National Research Council (CNR), Ancona; [3]Department of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy
E-mail: g.chemello@staff.univpm.it

The European sardine (*Sardina pilchardus*) is one of the most economically and ecologically relevant small pelagic species in the Mediterranean Sea.[1] In the Adriatic area, it represents the majority of annual landings, although, during the last years, fluctuations of total catches have been registered.[1] The decline of stock biomass is usually the consequence of high exploitation rates combined with fluctuations of environmental parameters that could affect food quantity and quality and consequently, the reproduction and health status of small pelagic stocks.[2] In particular, variations of environmental parameters could heavily affect the reproductive success of a species and consequently its survival. This study aimed to evaluate the current status of sardines' reproductive cycle with a particular focus on female gonads' development and maturation. Samples were collected once a month along the coast of the Marche region from April 2021 to March 2022. The identification of the ovary maturation stage, related to size class and time of the year, was performed to determine the actual sardines' reproductive season in the Adriatic Sea. Moreover, the histological analysis performed on the ovary highlighted the presence of different structural anomalies such as indefinite structures in the ooplasm, abnormal presence of atresia of both previtellogenic and vitellogenic oocytes and necrosis. The distribution of these anomalies varied according to age, size class and ovary maturation stage. These results suggest a possible alteration of the oogenesis process and the consequent malfunctioning of the ovary which could be identified as one of the causes of the population's decline.

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EXTRACTS FROM MARINE SPECIES MODULATE GLUCOSE UPTAKE AND CONSUMPTION BY HEPG2 CELLS

G. Abruscato, R. Tarantino, D. Ganci, A. Vizzini, M. Mauro, V. Arizza, M. Vazzana, C. Luparello

Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Palermo, Italy
E-mail: claudio.luparello@unipa.it

The marine environment with its enormous biodiversity represents an underexploited source of bioactive natural products.[1,2] The efficacy of preparations from marine invertebrates, *e.g.*, holothurians, and plants, *e.g.,* Posidoniacee, as anti-diabetic remedies is well-know from the traditional folk medicine. Hence, after the individuation of the species and their parts active on glucose metabolism, a detailed assessment of the cellular and molecular aspects of such beneficial property is imperative. In this work, HepG2 liver cancer cells which retain many differentiated hepatic functions[3] were grown in control conditions or exposed for 24 h to sublethal concentrations of aqueous extracts from cell-free coelomic fluid (CFE) of *Holothuria tubulesa*,[4] or either green leaves (GLE) or rhizomes (RE) of *Posidonia oceanica*,[5] with or without co-treatment with 10^{-7} M insulin. PAS staining, glucose consumption and glucose uptake assays showed that CFE and GLE, but not RE, determined the increase of intracellular glycogen accumulation and 2-NBDG internalization, this being a fluorescently-labeled deoxyglucose analogue, and the decrease of glucose present in cell medium, comparable to what observed with insulin treatment. No further synergistic effect was obtained in cases of insulin-extract co-exposures. Real time-PCR assays were performed to check the expression levels of genes coding for proteins involved in glucose uptake, *i.e., AKT, GLUT2, GLUT4, HNF1* and *IRS1* by HepG2 cells in the different experimental conditions. The preliminary data obtained suggest different molecular signalizations underlying the glucose-lowering properties of CFE and GLE. Further investigation will examine the actual intracellular accumulation of glucose metabolism-related factors and GLUT transporters, and the rate of translocation and exposure of the latter ones on the plasma membrane, in response to CFE and RE treatment.

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MACROSCOPIC AND MICROSCOPIC ANALYSES TO EVALUATE THE GONADIC MATURITY OF *ENGRAULIS ENCRASICOLUS* IN THE CAMPANIA COAST

M. Karam, D. Ciorciaro, R. Romano, F. Ferrigno, M. Peruzzi, L. Appolloni, G.F. Russo, P. Simoniello

Department of Science and Technologies, University of Naples "Parthenope", Naples, Italy
E-mail: myriam.karam001@studenti.uniparthenope.it

The Campania coast is an important spawning and nursery area for small pelagic fishes in Southern and Central Tyrrhenian Sea (GSA10). Anchovies, *Engraulis encrasicolus*, represent an impor-

tant fishery resource for coastal communities, even if in the last 5 years fishermen complain a decreasing trend in catches. A sampling program was developed to investigate biological aspects of the target stock. A key issue in the assessment of small pelagic fish stocks is the identification and classification of gonadic maturity by macroscopic and microscopic examination. Unfortunately, there is still a great deal of uncertainty concerning macroscopic identification, which remains to be better validated.[1] To assess the biological aspect of female reproductive cycle in the Gaeta, Naples and Salerno gulfs (the main fishing areas of purse seiners in Campania Region), samples from April to October were collected and macroscopically analysed to detect the maturity stages. Ovaries were fixed in 4% formalin and processed for histological investigation by light microscopy. Slides were stained with Haematoxylin-eosin to evaluate the oocyte developmental stages according to "the six-stage maturity scale".[2] Preliminary data showed that in June-July anchovies reached the peak of spawning season, as reported in literature.[3] In this period, oocyte development was morphologically represented by stage IV: the oocytes have reached the hydration, characterized by a rapid secretion of fluid by the granulosa cells of the follicle.[4] Further investigations are ongoing to establish the batch fecundity (peaks during the spawning season), the length at first maturity (L_{50}) and to detect possible differences among the fishing areas. The characterization of these aspects is essential to improve new approaches for the management of fisheries resources also based on variability in spatial distribution of target stock.

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HISTOLOGICAL ANALYSIS TO INVESTIGATE THE EFFECTS ON HEALTHY MUSCLE TISSUE AFTER IRRADIATION WITH FLASH THERAPY IN A MURINE MODEL

P. Simoniello[1], S. Belardo[1], U. Weber[2], A. Puspitasari[2], A. Abdollahi[3], C. Schuy[2], M. Durante[2], W. Tinganelli[2]

[1]Department of Science and Technology, University of Naples "Parthenope", Naples, Italy; [2]GSI Helmholtzzentrum für Schwerionenforschung, Biophysics Department, Darmstadt, Germany; [3]Clinical Cooperation Unit Radiation Oncology, Heidelberg Institute of Radiation Oncology, National Center for Tumor Diseases, Heidelberg University and German Cancer Research Center (DKFZ), Heidelberg, Germany
E-mail: salvatore.belardo002@studenti.uniparthenope.it

Radiotherapy is the main treatment for cancer diseases. However, the efficacy of radiation treatment has a limitation due to the toxicity in the surrounding normal healthy tissue, adverse side-effects are evident with consequence on the quality of life of the cancer patient. Recent studies are considering as a new strategy to treat cancer sparing normal tissue, FLASH therapy that use ultra-high dose rates radiation delivery.[1-2] For this purpose, in this work we studied the high-energy ^{12}C-ions delivered at an ultra-high dose rate in *in vivo* experiment using mouse as model system, to investigate the efficacy on tumour control and the impact on healthy muscle tissues. Mouse osteosarcoma LM8 cells were injected in the posterior limb of mice, then divided into three groups: FLASH dose-rate, conventional dose-rate, and sham irradiated. Healthy muscle tissues were processed for light microscopy. Results showed that irradiation with carbon ions was able to control the tumour, both at conventional and ultra-high dose rate. Therefore, FLASH decreases normal tissue toxicity, demonstrated by the reduced structural changes in muscle compared to conventional dose-rate irradiation. Histological analysis shows clear differences between FLASH and conventional irradiated animals in the healthy muscle tissue. Morphological disorganization compared to the normal structure of myofibril was observed, and a stronger alteration was detected in the muscle tissue after conventional dose-rate irradiation versus FLASH. Results reported in support a strong positive effect of FLASH irradiation with ^{12}C-ions. This new strategy could revolutionize the future of cancer treatment and widen the therapeutic window of radiotherapy.

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IN SITU PROTEOMICS: IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS DURING MOUSE FOLLICULOGENESIS

G. Fiorentino[1], A. Smith[2], G. Nicora[3], R. Bellazzi[3], F. Magni[2], S. Garagna[1], M. Zuccotti[1]

[1]Department of Biology and Biotechnology, University of Pavia, Pavia; [2]Department of Medicine and Surgery, University of Milano-Bicocca, Vedano al Lambro; [3]Department of Electrical, Computer and Biomedical Engineering, University of Pavia, Pavia, Italy
E-mail: maurizio.zuccotti@unipv.it

In the mammalian ovary, follicles go through a process of differentiation known as folliculogenesis. Mouse folliculogenesis proceeds through eight stages, from primordial types 1 and 2 (T1-T2) follicles to the preovulatory T8.[1] A deeper knowledge of the role of proteins in the cross-talk between oocytes, follicular cells, and the surrounding ovarian stroma would aid our understanding of the molecular mechanisms regulating folliculogenesis. MALDI mass spectrometry imaging (MALDI-MSI) is an innovative technology allowing to map directly *in situ* the complexity of macromolecules present in a tissue. In the ovary, MALDI-MSI has only been used to determine changes in the lipidome of follicular fluid and somatic cells during antral follicle maturation.[2,3] Here, we combine for the first time nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) with the spatial capabilities of MALDI-MSI to identify *in situ* the proteome of the prepubertal mouse ovary and to map the protein changes occurring during folliculogenesis. In the ovary, a total of 401 proteins were identified by nLC-ESI-MS/MS, 69 of which known to have an ovarian function.[4] The enrichment analysis of the 401 proteins revealed interesting KEGG/Reactome pathways, such apoptosis (11 proteins), developmental biology (11), PI3K-Akt (16), epigenetic regulation of gene expression (4) and extracellular matrix organization (6). Correlation of these proteins with the MALDI-MSI spatial information on 276 follicles, annotated in the entire ovary, allowed the protein profiles of individual follicle types to be mapped, revealing the presence of 94 proteins in T4-T8 follicles. When further analysed, 37 proteins showed a gradual quantitative change during follicle differentiation. Ten of these are known to play a role in follicle growth (NUMA1, TPM2), transition from the germinal vesicle to metaphase II oocyte (SFPQ, ACTBL, MARCS, NUCL), ovulation (GELS, CO1A2) and preimplantation (TIF1B, KHDC3). In conclusion, the spatial information provided

by MALDI-MSI indicated changes during folliculogenesis of proteins with a known function in the female gonad and of some whose role may be the subject of future studies.

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MITOCHONDRIAL DNA COPY NUMBER AS BIOMARKER OF HUMAN AND ENVIRONMENTAL HEALTH

G.S. Calogero[1], M. Giuga[1,2], V. D'Urso[1], V. Ferrito[1], A.M. Pappalardo[1]

[1]Department of Biological, Geological and Environmental Sciences, Section of Animal Biology "M. La Greca", University of Catania, Catania; [2]Institute for the Study of Anthropic Impact and Sustainability in the Marine Environment, IAS-CNR, Trapani, Italy
E-mail: giadacalogero@gmail.com

Unlike nuclear DNA (N), mitochondrial DNA (mtDNA) is particularly vulnerable to various types of damage due to the lack of protective histones and because it is in the proximity of ROS generation-sites within the electron transport chain.[1] As a result of the extreme sensitivity of the mitochondria to exposure to stressors, the number of copies of the mitochondrial genome (mtDNAcn) does not remain constant over time and it can be used as a biomarker of the exposure to various environmental contaminants. The mtDNAcn variation has been used as biomarker for the prognosis of different human diseases oxidative stress-mediated, but there is little data on the mtDNAcn as biomarker to assess environmental contamination.[2] Therefore, the aim of this study is to evaluate the effects of environmental pollutants on organisms living in polluted sites employing the mtDNAcn variation. A total of 20 samples of *Opsius heydeni* (Insecta, Hemiptera) were collected from two sites in eastern Sicily: Priolo Gargallo, affected by strong anthropic impact, and the Simeto River Oasis, as control site. The choice to investigate *O. heydeni* derives from the particular ecology of this species which is a sup-feeder on *Tamarix* spp., plants which are able to live on polluted sites accumulating in their leaves and bark high levels of heavy metals. The relative mtDNAcn was evaluated using qPCR of a portion of the mitochondrial COI and nuclear 18S genes. A decrease in the mtDNAcn in the specimens from the polluted site was observed. Similar results have been obtained in other species of invertebrates under different starvation and environmental pressures.[3,4] Further research on this topic comparing several model and non-model organisms could be useful to better understand the molecular mechanism of the mtDNAcn variation triggered by environmental stressors.

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EXPLORING THE EPITHELIAL-TO-MESENCHYMAL TRANSITION IN BREAST CANCER BY PROTEOMIC AND *IN SILICO* INVESTIGATIONS

E. Peri[1], M. Buttacavoli[1], C. D'Amico[1], S. Marino[1], F. Vaglica[1], I. Pucci-Minafra[2], E. Roz[3], S. Feo[1,2], P. Cancemi[1,2]

[1]STEBICEF Department, University of Palermo, Palermo; [2]Experimental Center of Oncobiology (COBS), Palermo; [3]Oncological Hospital La Maddalena, Palermo, Italy
E-mail: emanuela.peri@unipa.it

The epithelial-to-mesenchymal transition (EMT) is a biological process in which the epithelial cells lose their polarized phenotype to gain mesenchymal portraits. Essential in embryogenesis and wound healing process, the EMT also plays key roles in breast cancer (BC) progression, enabling cancer cells to acquire invasive and metastatic behaviour and enhancing cell survival.[1,2] In this study a proteomic profiling of Vimentin and E-cadherin expression, two master genes of the mesenchymal and epithelial phenotypes, was performed in 90 BC tissues by Western blotting. Beyond the high heterogeneity of expression of both markers, a surprisingly positive correlation was found between Vimentin and E-cadherin and different isoforms of both proteins were detected between patients. To better understand the biological significance of this heterogeneity, a bioinformatic approach was employed by using EMTome database[3] to unveil other specific EMT mediators in BC. The expression profiles and prognostic significance of selected genes was investigated by UALCAN and Kaplan-Meier Plotter databases, to obtain a cluster of differentially expressed genes between normal and BC tissues, significantly associated with prognosis. The obtained EMT mediators in BC were functionally classified using FunRich database and, interestingly, they were associated to Syndecan-2 activity, a proteoglycan with emerging roles in cancer progression. Indeed, it can have profound effects on tumor size, metastatic capability and overall patient survival rate. In conclusion, the obtained results suggest that the EMT in BC could be more complex than previously assumed and influence the cross talk between cancer cells and extracellular matrix. Additional studies are required to disclose the inter-connession between EMT and proteoglycans.

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TERATOGENIC EFFECTS OF DIMETHOATE ON EMBRYONATED EGGS *GALLUS GALLUS DOMESTICUS*

A. Salvaggio[1], E.M. Scalisi[2], R. Pecoraro[2], S. Indelicato[2], G. Giuffrida[2], F. Coppa[2], G. Coco[2], M.V. Brundo[2]

[1]Exp. Zooprophylatic Inst. of Sicily "A. Mirri", Palermo; [2]Department of Biological, Geological and Environmental Science, University of Catania, Catania, Italy
E-mail: antonio.salvaggio@izssicilia.it

Pesticides are useful to control any type of organism that attack and damage crops, as consequence their intensive use has raised considerable questions in the scientific field. Several studies have shown that the exposure to pesticides is a risk to the health of farmers, children and the environment.[1] Among pesticides, the organophosphates are anticholinesterase insecticides widely used

in agriculture. In this work, embryonated eggs of *Gallus gallus domesticus* were used to evaluate the toxicity of the Dimethoate, a acetylcholinesterase inhibitor. A stock solution of dimethoate 0.04 g/10 mL, was used to obtained the working solutions: 0.004 g/10 mL, 0.0004 g/10 mL and 0.00004 g/10 mL. The solutions were inoculated into the fertilized chick eggs by insulin syringe and placed in an incubator, we have included also control samples. On 5th, 10th and 19th day after incubation, window was made in the shell to taken the embryos, thus they were fixed for histological analyses. We have observed that the treated samples were viable, but showed evident anomalies such as: visceral ectopia, caudal hernia and lack of resorption of the yolk sac. The histological analyzes have highlighted alterations in the liver, intestine and lungs tissue. Therefore, it is evident that exposure to dimethoate leads to an increased risk of teratogenic effects during embryonic development.

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BOVINE MILK EXTRACELLULAR VESICLES: AN ORAL DRUG DELIVERY SYSTEM FOR BIOACTIVE COMPOUNDS

S. Tacconi[1], F. Vari[2], L. Buccini[3], S. Sennato[2], S. Dinarelli[5], M. Zuccotti[6], L. Dini[7,8]

[1]Inrae Carmen Lab. Lyon, France; [2]DFFVE Sapienza University of Rome, Rome, Italy; [3]SBAI, Sapienza University of Rome, Rome, Italy; [4]ISC, CNR Rome, Italy; [5]ISM, CNR Rome, Italy; [6]DBBLS, University of Pavia, Pavia, Italy; [7]DBBCD, Sapienza University of Rome, Rome, Italy; [8]CNIS, Sapienza University of Rome, Rome, Italy
E-mail: stefano.tacconi@inrae.fr

Extracellular vesicles (EVs) are now be isolated by almost all biological fluid, and their isolation by raw bovine milk (MEVs) represents a low-cost source of extremely biocompatible EVs, that are receiving great attention as a new drug delivery system.[1,2] Thus, our work is focused on the optimization of the isolation method of EVs from raw cow milk, in consideration of the presence of casein micelles, which having similar EVs size, are posing a severe obstacle to isolation efficiency.[3] We found that the best approach for EVs isolation from bovine milk was the enzymatic treatment combined with size-exclusion chromatography. Our choice is corroborated by the results of the extensive characterization performed with different methods: Western blots, high-resolution microscopy, cryoTEM and dynamic light scattering together with thin-layer chromatography for the profile of the lipid composition of MEVs. In fact, the enzymatic removal of casein combined with SEC provided the best results in terms of higher purity, unaltered EVs quality, and homogeneous size. MEVs were passively loaded with curcumin (CurMEVs) and, by exploiting the intrinsic optical and fluorescence properties of curcumin, loading efficiency, stability, and solubility were determined. CurMEVs morphology, integrity, and loading efficiency were also evaluated under different storage conditions and pH levels over time. 3 h of incubation of curcumin with MEVs represents the maximum loading efficiency and CurMEVs shows greater stability and solubility than free curcumin. CurMEVs integrity is maintained when stored at low temperature and exposed to varying pH levels. The feasibility of CurMEVs as oral drug delivery system was assayed in a 2D *in vitro* intestinal barrier model. All the tested parameters, *i.e.* toxicity of CurMEVs, integrity of epithelial layer and transepithelial crossing ability, confirm the biocompatibility of MEVs and the ability to entering the cells and slowly discharging the cargo.

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DOES MICROPLASTICS INDUCE SUFFERENCE IN *XENOPUS LAEVIS* EMBRYOS?

F. Melfi, V. Impagliazzo, C. Artiaco, M.G. Orsi, C. Fogliano, R. Carotenuto

Laboratory of Developmental Biology, Department of Biology, University of Naples Federico II, Italy
E-mail: rosa.carotenuto@unina.it

Microplastics (MPs), originating from industrial and household products or from the degradation of larger plastics, are nowadays pollutants of global concern. These particles are found in aquatic environments in high concentrations, up to thousands of particles per m^3, and may adversely affect aquatic organisms.[1,2] The ability of MPs to adsorb pollutants and subsequently liberate them into marine and freshwater systems is an additional concern.[3,4] Since *Xenopus laevis* embryos are a good model to study the effects of MPs accumulation, in this study, through FETAX assay[4], commercial polystyrene MPs of 1 and 3 μm were tested at 0.1, 1 and 10 mg/L concentration, to check for any adverse effect on embryonic development. The impact on heart rate, the influence on the expression of some early developmental genes and pro-inflammatory cytokines were investigated, together with ROS production. The localization and effects of MPs were analyzed with light and electron microscopy. Results indicate that microplastics cause a low mortality rate, reaching a maximum of 30% (3 μm at 10 mg/L). Embryos grow normally, except for those treated at 10 mg/L with 3 μm MPs, probably due to the ingestion of MPs which, as highlighted by microscopy investigation, would accumulate and cause significant harm to the intestinal epithelium. MPs induced a low malformation rate, even though very severe, and increased production of ROS[4]; moreover, levels of *bmp4, fgf8*, *pax6* and *rax1*, master genes in embryonic development, were modified. Finally, the induction to an inflammatory state was supposed, considering the increased expression of *tnfa* and *il1b*. Our data indicate that the effects caused by MPs certainly depend on their size, but that concentration is the most important aspect to consider. Therefore, it is of fundamental importance to reduce the production of these substances and free the water from the already existing ones.

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MARINE MACROALGAE DIETARY SUPPLEMENTATION PROVIDES GENOPROTECTION IN FISH (*DIPLODUS SARGUS*) AGAINST INORGANIC MERCURY: EVIDENCES FROM ACCUMULATION LEVELS IN BLOOD AND ERYTHROCYTIC NUCLEAR ABNORMALITIES

A.C. Neto[1], F. Brandão[1], R. Cesário[2], A. Marques[1],V. Pereira[1], M. Pacheco[1], P. Pereira[1]

[1]CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal; [2]Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal
E-mail: acneto@ua.pt

The benefits of marine macroalgae have been largely explored with a focus on human health, while advantages to fish condition remain elusive. Some of the benefits have been associated with bioactive compounds of macroalgae, including genoprotection. This study addressed the genoprotection afforded by a macroalgae-enriched diet to fish (*Diplodus sargus*) exposed to waterborne inorganic mercury (iHg). For this purpose, fish were fed during 3 months with a macroalgae-enriched feed [total incorporation of 5%, with *Ulva rigida, Fucus vesiculosus* and *Gracilaria gracilis*, equitably represented - algae supplementation (A)], while non-supplemented fish were fed with a standard diet (S). Then, both dietary background groups were exposed to waterborne iHg (2 μL L^{-1}) for 7 days (T7) (groups AHg and SHg), followed by a post-exposure period of 14 days (PE14). Control fish, unexposed to iHg, were maintained over the experiment (AC and SC). At T7 and PE14, fish of the different groups (AC, SC, AHg, SHg) were sacrificed and blood was collected for the determination of total Hg levels and for the assessment of genotoxicity-related parameter (erythrocytic nuclear abnormalities (ENA)). Fish with a macroalgae-diet background accumulated lower levels of Hg in the blood that those under a standard diet. In line, the ENA results suggested a decrease of iHg genotoxicity at the group feed with the macroalgae-enriched diet. Results are promising by revealing the genoprotection of a macroalgae dietary supplementation against the genotoxicity of iHg in fish erythrocytes.

PEROXISOMAL ALTERATIONS IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

M. Terricola[1,2], R. Price[1,2], S. Scaricamazza[3], I. Salvatori[3], S. Giuliani[1], C. Fiorucci[1], M. Cervelli[1,2], F. Berardinelli[1,2], A. Ferri[3], C. Valle[3], S. Moreno[1,2]

[1]Department of Science, University Roma Tre, Rome; [2]Lab. of Neurodevelopmental Biology, Neurogenetics and Molecular Neurobiology; [3]Lab. of Neurochemistry, IRCCS Fondazione S. Lucia, Rome, Italy
E-mail: martina.terricola@uniroma3.it

Peroxisomes are dynamic organelles, playing a crucial role in numerous anabolic and catabolic functions and able to respond to physiological and pathological changes, by altering their enzyme content, morphology and abundance.[1] Such features are controlled by peroxisome proliferator activated receptors (PPARs), whose transcriptional regulatory action depends upon binding to their ligands and is regulated by coactivators like PGC1α. Importantly, peroxisomes biogenesis pathways are shared with mitochondria. Noteworthy, these types of organelles cooperate in multiple metabolic and signalling networks, such as lipid (*e.g.*, fatty acids β-oxidation) and reactive oxygen species (ROS) metabolism. Consistently, peroxisomal alterations can influence mitochondrial functions, and *vice versa*.[2] Mitochondrial dysfunction, associated with increased energy expenditure and oxidative stress, hallmarks amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting motor neurons and leading to paralysis. Skeletal muscle plays a pathological role in ALS, contributing to defective energy metabolism and determining a derangement of basal metabolic rate, switching its metabolism towards an oxidative phenotype and to the preferential use of fatty acids as fuel.[3] In this study we addressed peroxisomal involvement in ALS onset and progression, by analyzing the expression of peroxisomal markers in the spinal cord and gastrocnemius of *SOD1^{G93A}* transgenic mouse model at different stages. Immunoblotting and qRT-PCR analyses show a significant increase of fatty acyl β-oxidation enzymes (thiolase, ACOX1), antioxidant enzymes (catalase, SOD2), at the symptomatic stage. Such upregulation is accompanied by PMP70 and PPARα induction. Overall, our data strongly suggest a peroxisomal involvement in the disease, in both muscle and nervous tissue, which deserves further attention.

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YOLK INTERNALIZATION PATTERNS IN EMBRYOS OF LOGGERHEAD SEA TURTLE (*CARETTA CARETTA*): HOW NUTRIENTS ARE TRANSFERRED TO THE EMBRYOS?

E. Trotta[1], G. Chemello[1], L. Papetti[2], L. Di Renzo[3], M. Matiddi[4], C. Silvestri[4], O. Carnevali[1], G. Gioacchini[1]

[1]Department of Life and Environmental Science, Polytechnical University of Marche, Ancona; [2]CRTM "tartAmare", Marina di Grosseto; [3]Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo; [4]ISPRA, National Institute for Environmental Protection and Research, Rome, Italy
E-mail: trotta.erica@gmail.com

The embryogenesis of Loggerhead sea turtles (*Caretta caretta*) is strictly related to the energy sources stored in yolk necessary to sustain all the developmental processes until hatching. The yolk molecular composition and its internalization pathway is still unclear in sea turtle. The scarce information available in reptiles suggested that the yolk is composed mainly of lipids, proteins, carbohydrates and some inorganic ions.[1] More in detail, in sea turtles, previous studies highlighted the protracted presence of lipidic compartment in yolk until last developmental stages proposing a selective absorption pathway of yolk components.[2] To fill the gap, this study evaluated the nutrients transport pathway from the yolk to the liver. Unhatched embryos at different developmental stages were collected in 4 different nests laid in early and late reproductive season in 2021 along the north-western Mediterranean coast (Tuscany, Italy). Embryos were sampled and yolks and livers were properly processed and analysed through SDS-page. The electrophoresis patterns highlighted differences in protein composition of yolk from embryos at different developmental stages reflecting an internalization patterns of yolk component in liver. Concomitantly, slight differences have been evidenced comparing yolk composition of early or tardive eggs. This preliminary study represents a first analysis of the yolk absorption mechanism during the whole embryonic development of Loggerhead sea turtles,

clearing up the role of the liver during yolk absorption.

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CONSERVED ROLE OF SEROTONIN IN MELANOGENESIS OF CHORDATES

R. Pennati[1], S. Candiani[2], M. Bozzo[2], A. Pennati[3], L. Morabito[1], S. Mercurio[1]

[1]Department of Environmental Science and Policy, University of Milan, Milan, Italy; [2]Department of Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy; [3]Institute of Zoology and Center of Molecular Biosciences, University of Innsbruck, Innsbruck, Austria
E-mail: roberta.pennati@unimi.it

Recently scientific attention has been payed to unravel the role of serotonin (5HT) in melanogenesis, for its possible involvement in human skin diseases such as vitiligo or hyperpigmentation.[1] Human skin cells express serotonin receptors including 5-HTR1A, 5-HTR1B, 5-HTR2A, 5-HTR2B, 5-HTR2C, and 5-HTR7.[2] In vertebrates, melanin pigments are most often produced by melanocytes, which are dendritic cells derived from embryonic neural crest cells.[3] Melanin synthesis from the precursor L-Tyrosine is a complex biochemical process that requires the enzyme tyrosinase (TYR) and two tyrosinase-related proteins, TRP-1 and TRP-2, which are structurally related to tyrosinase and share ~ 40% amino acid homology.[4] The role of 5HT in melanogenesis was investigated by pharmacological treatments in different vertebrate species and in human cell cultures. 2,5-dimethoxy4-iodoamphetaminehydrochloride (DOI), a 5-HTR2A agonist, increased melanin content and TYR activity in SK-MEL-2 cells in a dose-dependent manner. Fluoxetine, a selective serotonin reuptake inhibitor, up-regulated melanin synthesis in human melanocytes and in zebrafish and its effects were inhibited by WAY-100635, a selective 5-HTR1A antagonist.[5] To test if the role of serotonin in melanogenesis predates the emergence of vertebrates, we exposed embryos of the ascidian *Ciona intestinalis* to WAY-100635 and analyzed the effects on genes involved in melanogenesis. We found that treatments caused a drastic decrease in pigment content in sensory organs compared to controls but they had no effects on *Ci-Tyr* and *Ci-Tyrp 1/2* expression. Instead, we found a perturbation of *Ci-TCF*, a downstream factor of the canonical Wnt pathway, known to play a role in pigment cell terminal differentiation in *C. intestinalis*. Our results suggest that the role of serotonin in melanogenesis is conserved in olfactores, the group that includes tunicates and vertebrates, even though the specific molecular mechanisms require to be further explored.

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THE ROLE OF THE ZEBRAFISH PROTEIN AMBRA1B ON THE PRIMORDIAL GERM CELL SURVIVAL AND SEX DETERMINATION

C.M. Fontana[1], F. Terrin[1], M. Caichiolo[1], R. Verin[2], F. Nazio[3], O. Carnevali[4], F. Cecconi[5], L. Dalla Valle[1]

[1]Department of Biology, University of Padua, Padua; [2]Department of Comparative Biomedicine and Food Science (BCA), University of Padua, Padua; [3]Department of Biology, University of Rome Tor Vergata, Rome; [4]Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona; [5]Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy
E-mail: luisa.dallavalle@unipd.it

AMBRA1, initially identified as a positive regulator of the Beclin1-dependent autophagic pathway, is an intrinsically disordered protein working as a scaffold-molecule to coordinate many cellular processes, such as autophagy, apoptosis, cell proliferation and development. The zebrafish genome contains two *ambra1* paralogous genes (*a* and *b*), both involved in neural, muscle, and cardiac development. Furthermore, both genes are expressed at high levels in the brain and gonads. Unlike the knockdown results, the mutants of both lines show no obvious developmental defects. However, all homozygous *ambra1b* mutants develop as fertile males. In this study, we demonstrated that silencing of the *ambra1b* gene results in a reduction of the primordial germ cells (PGCs) number, a condition that, in zebrafish, leads to the development of all-male progeny. PGCs reduction was confirmed by knockdown experiments with *ambra1b* ATG and splicing morpholinos. Moreover, the PGC number can be rescued by injection of ambra1b and human *AMBRA1* mRNAs, but not *ambra1a* mRNA. PGC loss was also rescued by injection with human *AMBRA1* mRNA mutated in the PP2A, LC3, and TRAF6 binding sites but not with the transcripts mutated in the CUL4-DDB1 binding region, thus suggesting that the interaction with this complex is involved in PGC protection from loss. The results from zebrafish embryos injected with murine *Stat3* mRNA and *stat3* morpholino suggest that Ambra1b could indirectly regulate this protein through CRL4-DDB1 interaction. Finally, the reduced reproductive capabilities of both mutant zebrafish lines and the pathological alterations, including tumors, mainly limited to the gonads reveal that Ambra1 functions are not limited to sex determination, supporting a role in the regulation of reproductive physiology.

RELATIVE POTENCY AND MIXTURE EFFECTS OF TWO BISPHENOL PLASTICIZERS (BPA AND BPB) ON *XENOPUS LAEVIS* DEVELOPMENT

E. Menegola[1], F. Metruccio[2], M. Battistoni[1], R. Bacchetta[1], F. Di Renzo[1]

[1]Department of Environmental Science and Policy, University of Milan, Milan; [2]ICPS, ASST Fatebenefratelli Sacco, Milan, Italy
E-mail: elena.menegola@unimi.it

Bisphenol A (BPA) is a plastic additive with endocrine disruptive activity, classified in 2017 by EU ECHA as substance of very high concern. A correlation between environmental exposure to BPA and congenital defects has been described in humans and in experimental species, including the amphibian *Xenopus laevis*. Among BPA analogues, bisphenol B (BPB) was introduced as BPA-alternative molecule in different not-EU countries, including US, but

seems to share with BPA its endocrine disruptor properties. No data are available on developmental toxicity of BPB and on effects related to BPA-BPB combined exposure. Aim of the present work is the evaluation of the effects of the single exposure to BPA and BPB or of their mixture in a *X. laevis* development model. R-FETAX was applied, with the exposure covering the first day of development (corresponding to the phylotypic developmental period, NF stages 9-26) and development maintained during the next 5 days (till NF stage 46 reached in historical controls). Samples were monitored for lethal effects during the full six-day test period. At the end of the test, the external morphology was evaluated. Total effects were modelled using PROAST software: BPA and BPB dose-relation curves were obtained and the relative potency factor (RPF) of BPB versus BPA was derived. Mixture effects were also described by modelling. Overall data modelling showed BPB relative potency about 3 times higher than BPA. Mixture effects suggested a common mode of action of the two molecules.

SPERMATOGENESIS AND MITOCHONDRIAL FUNCTIONALITY IN HIGH FAT DIET-FED RATS

S. Falvo[1], D. Latino[1], A. Santillo[1], M.M. Di Fiore[1], M. Venditti[2], G. Chieffi Baccari[1]

[1]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, and [2]Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Caserta, Italy
E-mail: sara.falvo@unicampania.it

In this study, we investigated the cellular response induced by a high fat diet (HFD) in rat testis, focusing on the mitochondrial compartment. After five weeks of HFD, an increase in both testicular levels of malondialdehyde and reduced form of glutathione and, a decrease in both SOD2 and catalase indicated an increase in oxidative stress due to enhanced lipid peroxidation. The results showed also that excessive ROS production triggers apoptosis and alters spermatogenesis. Particularly, significant lower expression levels of SYCP3, an essential structural component of the synaptonemal complex, in spermatocytes and viceversa higher levels of PCNA, a nuclear antigen of cell proliferation, in spermatogonia were observed in HFD-fed rats as compared to control animals. It has known that spermatogonia use glycolysis for self-renewal and proliferation, then energy production is shifted from glycolysis to mitochondrial respiration in spermatocytes and spermatids.[1] In this regard, a strong mitochondrial damage were detected in the testis of HFD-fed rats. Specifically, an induction of mtDNA damage/repair and, a decrease in both mtDNA copy number and expression of the POLG, enzyme involved in mtDNA replication, occurred in the HFD-testis. Finally, our results showed an enhanced mitophagy which was not compensated by mitochondria biogenesis. Consistently, a reduction in mitochondria number, as evidenced by the decrease in the protein expression of TOM20, a mitochondrial outer membrane receptor, was detected. Therefore, our results suggest that the HFD-induced mitochondria damage primarily affects the normal progression of meiosis in spermatocytes. The increased expression of PCNA in spermatogonia, indicative of increased proliferative activity, could be a compensatory response to the impaired maturation of germ cells.

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LONG-TERM *IN VITRO* DYNAMIC CULTURE OF BOVINE OVARIAN CORTICAL TISSUE (BOCT) INCREASES FOLLICLE GROWTH, VIABILITY AND AMH SECRETION

V. Barbato[1], V. Genovese[1,2,3], V. De Gregorio[1], A. Travaglione[1], A. Candela[1], A. Amoresano[2], S. Sepico[2], G. Pinto[2], G. Mondrone[3], T. Dhooghe[4], S. Longobardi , W.Zheng[4], G. Catapano[5], R. Gualtieri[1], R. Talevi[1]

[1]Department of Biology, University of Naples Federico II, Italy; [2]Department of Chemical Science University of Naples Federico II, Italy; [3]IVF RED srl, Caserta, Italy; [4]Merck KGaA, Darmstadt, Germany; [5]Department of Environmental & Chemical Engineering, University of Calabria, Cosenza, Italy
E-mail: barbato_vincenza@libero.it

Short-term ovarian tissue culture is the first step of *in vitro* folliculogenesis procedures aimed to obtain healthy secondary follicles necessary for further growth and generation of mature oocytes.[1] However, the yield and health of secondary follicles remain limited. We previously reported that dynamic *in vitro* culture of ovarian cortical tissue in a novel perifusion bioreactor (PB) (patent 102020000027290) enhances follicle growth and health compared to conventional static culture (CD).[2] In the present study, long-term culture of BOCT was attempted to preserve tissue health and obtain more competent secondary follicles. BOCT slices (1x1x0.5 mm) were cultured in group of 10 for 14 days in PB and CD, monitoring whole tissue viability (LDH) and AMH secretion in spent media every 2 days. Follicle stages and quality were assessed through histology and live-dead confocal analysis, respectively at the end of culture. Data (939 follicles) indicated that culture in PB *vs* CD increases the secondary follicle rate (21.2 *vs* 8.2%, $p<0.05$), and follicle quality and viability (grade I/II, 70 *vs* 18.9%, grade III, 30 *vs* 81.1%; live follicles 62.6 *vs* 35.3%, p< p0.01). LDH activity in PB was markedly lower than in CD at all culture times (PB14d 150.5 nmol *vs* CD14d 233.1 nmol). AMH secretion continuously increases in PB whereas in CD it markedly decreases (1700 *vs* 67 picomol/Vtot). Findings indicate that the increased transport of solutes and dissolved oxygen, and the biomechanical stimulation in our novel dynamic PB play a key role during BOCT culture. Analysis of LDH and AMH levels in spent media confirm that dynamic PB better maintains general tissue health and promote a better functionality of growing follicles.

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ESTABLISHING INDIVIDUAL BOVINE EMBRYO CULTURES IN SUB-MICROLITER ENVIRONMENT MAINTAINS THE BLASTOCYST COMPETENCE

V. De Gregorio[1], A. Travaglione[1], V. Genovese[1], A. Candela[1], V. Barbato[1], T. Dhooghe[2], S. Longobardi[2], R. Talevi[1,3], R. Gualtieri[1]

[1]Department of Biology, University of Naples Federico II, Naples, Italy; [2]Merck KGaA, Darmstadt, Germany; [3]IVF RED srl, Caserta, Italy
E-mail: vincenza.degregorio@unina.it

In ART, human embryos are cultured either in groups in relatively large volumes, or individually in drops under mineral oil. However, such static culture conditions fail to mimic the physio-

logical dynamic microenvironment experienced by human embryos in the oviduct.[1,2] Microfluidic dynamic culture systems in which embryos are cultured singularly or in group in sub-microliter volumes could improve blastocyst rates and competence.[3] The development of dynamic devices for single embryo culture able to monitor oxygen and other relevant metabolites through sensors is strictly dependent on the embryo ability to develop in extremely reduced media volumes. In this work, we aimed to examine the effects of the sub-microliter environment and embryo density on developmental rates and embryo quality using the bovine as animal model due to the high analogies with human embryo development. An extensive characterization of the embryo developmental rates and competence was carried out measuring blastocyst rates, blastomeres number, and apoptosis under the following culture conditions: a. conventional group embryo culture -CGEC 50/well; embryo density 1/10 μL; b. Microwell single embryo culture in 70 nL -MSEC 16/chamber; embryo density 1/70 nL; c. Microwell group embryo culture -MGEC 16/chamber; embryo density 1/3.75 μL. Our preliminary results showed that: i) individual culture in 70 nL does not perturb embryo development in comparison with MGEC; ii) a lower number of DNA fragmented blastomeres is observed in blastocysts developed in MSEC and MGEC in comparison with CGEC. The proof that confined single embryo culture in extremely reduced volumes does not impair blastocyst rates and quality is a pre-requisite for the design of microfluidic sensorized devices in which embryos are cultured and monitored singularly to identify highly competent blastocysts for single embryo transfer.

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EFFECTS OF DIBUTYL PHTHALATE AND ENDOGENOUS SEX HORMONES ON HUMAN PROSTATE CELLS

A. Mileo[1], T. Chianese[1], M. Licata[1], V. Laforgia[1,2], M. De Falco[1,2]

[1]Department of Biology, University of Naples Federico II, Naples; [2]National Institute of Biostructures and Biosystems (INBB), Rome, Italy

E-mail: madefalco@unina.it

Reproductive health is a sensitive target of Endocrine Disrupting Chemicals (EDCs), since these substances can interfere with normal sex steroid balance. Phthalates, one group of EDCs, are ubiquitous in the environment because they are widely used as solvents, additives, and plasticizers in various consumer goods. Dibutyl phthalate (DBP) is a low-molecular weight phthalate, found in almost all personal care products, cosmetics, printing inks, children's toys, and pharmaceutical coatings. People are constantly exposed to phthalates in air, food, water, and contact with a variety of consumer products. Phthalates have agonistic and antagonistic effects. Several *in vivo* studies have shown a direct relationship between an increase in the incidence rate of prostatic pathologies and phthalate exposures. Because the prostate gland reacts to sex-steroids, their regulation -singly or in combination- is important for the gland's development and function. Androgens can promote the growth factors of cell proliferation by interacting directly with the androgen receptor (AR). While serum androgen levels decrease during aging, the levels of 17β-estradiol (E_2) remain constant, and they increase the E_2:TT ratio. In the present work, we studied the effects of DBP on human prostate epithelial cells (PNT1A) with or without sex endogenous hormones, testosterone (T) and E_2, to assess the potential synergistic or antagonistic effects of DBP. First, we observed cell viability showing that all mixtures increased cell proliferation, with a potential antagonistic effect of DBP. Second, we observed the localization of estrogen (ER) and AR receptors, and we showed that DBP, alone or in combination with T or E_2 or both (T+E_2), was able to interact with steroid receptors and to translocate them into the nucleus, even if in times different from endogenous hormones. Furthermore, we performed a mobility scratch assay and we showed that DBP decreased cell migration velocity, while in mixture its effect seemed like to hide by endogenous hormones. In conclusion, we have shown that the mixtures containing DBP induced a strong imbalance of prostate cell physiology.

TITANIUM DIOXIDE NANOPARTICLES: TOXICITY ON MALE REPRODUCTIVE SYSTEM

E.M. Scalisi[1], R. Pecoraro[1], A. Salvaggio[2], F. Capparucci[3], G. Impellizzeri[4], M.V. Brundo[1]

[1]Department of Biological, Geological and Environmental Science, University of Catania, Catania; [2]Exp. Zooprophylatic Institute of Sicily "A. Mirri", Palermo; [3]Department of Biological Veterinary Sciences, University of Messina, Messina; [4]CNR-IMM, Catania, Italy

E-mail: elenamaria.scalisi@unict.it

Developing countries have seen a reduction in male reproductive parameters (sperm quality and Leydig cell function). Male infertility is generally linked to xenobiotics with hormonal activity called Endocrine Disruptors (IE) that compromise reproduction. Nowadays, the nanoparticles (NPs) are among the exogenous substances present in the environment because they can be released into the environment as a result of their applications.[1] Titanium dioxide nanoparticles (TiO_2-NPs) are the most versatile category of nanoparticles, as consequence living beings are exposed continuous and involuntary to TiO2-NPs.[2] In this work, adult males of *Danio rerio* were exposed to TiO_2-NPs for 30 days to evaluate their toxicity on male reproductive system. Randomly the fishes were divided into 4 groups of 10 fishes: 3 experimental groups (1 mg/L, 2 mg/L and 4 mg/L TiO_2-NPs) and control group (only osmosis water). Daily the fishes were monitored and at the of the exposure, they have been euthanized by anesthesia and the testes were dissected. Protocols for preparation of histological, immunohistochemical and semithin sections were carried out on testis; moreover expression of marker genes by the real-time quantitative PCR (qRT-PCR) was evaluated. We have observed that the TiO_2-NPs caused an alteration of the morphological/structural organization of the gonad. The immunofluorescence investigation showed a positivity for the SHBG and P540 biomarker. SHBG protein acts as a carrier of androgens and estrogens regulating their bioavailability. The results of qRT-PCR showed an increase in the gene expression of the genes involved in oxidative stress and also of the gene responsible for the conversion of testosterone to dihydrotestosterone. Since Leydig cells are mainly involved in this activity, an increase in gene activity not ruled out the ability of TiO_2-NPs to act as endocrine disruptor.

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THE HSP70 EXPRESSION AS CYTOPROTECTIVE RESPONSE AGAINST GENETICS AND OXIDATIVE DAMAGE ON HUMAN SPERMATOZOA EXPOSED TO POLYSTYRENE NANOPARTICLES

M. Contino

Department of Biological, Geological and Environmental Sciences, University of Catania, Catania, Italy
E-mail: martina.contino@phd.unict.it

The survival of sperm cells requires a series of parameters (pH, antioxidant substances, fructose, *etc.*) that allow the maintenance of an optimal microenvironment. The presence of pollutants, including nanoplastics, could perturb this environment due to possible interactions with the spermatozoa, affecting the main parameters, crucial for successful fertilization. The aim of this work was to highlight the potential effects of polystyrene nanoplastics (50 and 100 nm) at increasing concentrations (0.1 - 0.5 - 1 μg/mL) on the metabolism of human spermatozoa and the possible responses induced to counteract the damage. In detail, the expression of HSP70 proteins and the correlation with the change in several parameters, such as DNA fragmentation, increased oxidative stress and decreased mitochondrial activity, were analyzed. The results obtained show an increase in structural and metabolic damage in samples exposed to nanoplastics with a diameter of 50 nm; less damage was recorded in samples exposed to nanoplastics with a larger diameter. In samples exposed to 50 nm nanoplastics, stimulation of HSP70 expression was also evident, especially at the level of neck of spermatozoa. Correlation analysis also showed less impairment in cells with increased HSP70 expression. The results underline the well-known danger of nanoplastics on biological systems and show the sensitivity of spermatozoa to their presence, underlining various structural and metabolic damages and highlighting a damage recovery mechanism.

MULTISENSORY REPRESENTATION OF IMPRINTED COURTSHIP CUES

S. Zucca[1,2], A. Stella[1,2], N. Mancin[1,2], C. La Rosa[1,2], P. Peretto[1,2], S. Bovetti[1,2]

[1]Department of Life Sciences and Systems Biology, University of Turin; [2]Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Orbassano, Italy
E-mail: serena.bovetti@unito.it

Animals evolved a wide range of approaches to attract opposite-sex partners, and most of these strategies rely on the integration of courtship cues across multiple sensory modalities. In rodents, male mice attract females using a combination of olfactory (*i.e.*, pheromones and molecules contained in their urine and scents) and acoustic (*i.e.*, ultrasound vocalizations, USVs) cues. Past works demonstrated that, during early life, females establish memories of both odors and sounds of their father and siblings, a process called sexual imprinting. Females can later recall these imprinted memories to find the most suitable partner, avoiding mating with their close relatives and reducing inbreeding.[1,2] Here we focus on the brain representation of sexual odors in adult females and compare how the brain represents imprinted versus unfamiliar opposite-sex olfactory cues. By using whole-brain immunolabeling of immediate early genes (cFos) as a proxy of neuronal activation, iDISCO tissue clearing, and light-sheet microscopy, we identified several brain regions activated by both imprinted and unfamiliar odors and, interestingly, a subset of hypothalamic areas which were activated only by unfamiliar cues. By looking at the correlations across brain regions, we further identified different subnetworks recruited under the two conditions.[3] Topological analysis of these identified subnetworks will reveal key areas coordinating as hubs in response to the olfactory cues. Finally, we started investigating if opposite sexual odors can modulate neuronal responses to male vocalizations in the primary auditory cortex (AUCx) of female mice. We are currently performing *in vivo* intracellular recordings from AUCx in awake females exposed to male olfactory and/or acoustic cues. These results will provide novel insights into how the brain integrates multiple sensory cues involved in courtship and mate behavior, and how their representation is affected by the post-natal experience.

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AGE-DEPENDENT FUNCTIONAL CHANGES IN MEMBRANE TRANSPORT SYSTEMS: THE KEY ROLE OF ANTIOXIDANTS

S. Zucca[1,2], A. Stella[1,2], N. Mancin[1,2], C. La Rosa[1,2], P. Peretto[1,2], S. Bovetti[1,2]

[1]Department of Life Sciences and Systems Biology, University of Turin; [2]Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Orbassano, Italy
E-mail: serena.bovetti@unito.it

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AGE-DEPENDENT FUNCTIONAL CHANGES IN MEMBRANE TRANSPORT SYSTEMS: THE KEY ROLE OF ANTIOXIDANTS

S. Spinelli[1], A. Remigante[1], A. Marino[1], S. Dossena[2], M. Morabito[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy; [2]Institute of Pharmacology and Toxicology, Paracelsus Medical University, Salzburg, Austria
E-mail: saspinelli@unime.it

Aging is a pathophysiological process closely related to increased oxidative stress (OS) that induces several changes in cells and tissues, resulting in an increased risk of disease and death.[1,2,3] The aging-induced injury could affect membrane transport, which is essential to ensure cellular homeostasis. Therefore, the purpose of this work focuses on the activity of two transport systems, the erythrocyte Band 3 protein (AE1) and Kv3.1 channel, in an aging model induced by the exposure to D-Galactose (D-Gal) and evaluate the beneficial effect of two antioxidant substances: açai berries extract and melatonin. Band 3 protein exchange capability was evaluated by the rate constant of SO_4^{2-} uptake. Kv3.1 activity was monitored by whole-cell patch-clamp technique on the endogenous (HEK293 Phoenix) and ectopic (NIH/3T3 fibroblasts) currents. Cell viability as well as markers of OS, including thiobarbituric acid reactive substance (TBARS) levels, total content of sulfhydryl (SH) groups, and the intracellular oxidative assessment (reactive oxygen species (ROS) production, superoxide dismutase (SOD) and catalase (CAT) activity, GSH content) have been analysed. Our results show that: i) aging affects membrane transport, thus altering anionic exchange capability through AE1 and Kv3.1 current; ii) these changes are associated with increased oxidative stress at both membrane and intracellular levels; iii) açai berries extract and melatonin counteract the changes induced by aging-related oxidative stress, thus restoring the functionality of AE1 and Kv3.1. These data contribute to clarify the molecular mechanisms of cellular aging and further suggest that antioxidant strategies might be useful to counteract aging-related diseases. Further studies are needed to verify the molecular pathways that impair membrane transport activity.

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AQUAPORIN DEFICIENCY AFFECTS MORPHOLOGICAL AND GLYCOSYLATION PATTERNS IN THE GASTRIC CELLS OF A MURINE MODEL

D. Mentino[1], P. Nicchia[1], A. Frigeri[2], S. Desantis[3], M.V. Guglielmi[1], D. Semeraro[1], G. Scillitani[1], M. Mastrodonato[1]

[1]Department of Bioscience, Biotechnology and Environment; [2]School of Medicine, Department of Translational Biomedicine and Neuroscience; [3]Department of Precision and Regenerative Medicine and Ionian Area, Section of Veterinary Clinics and Animal Productions, University of Bari "Aldo Moro", Bari, Italy
E-mail: donatella.mentino@uniba.it

Aquaporins (AQPs) are involved in the regulation of water transports across the epithelia of the gastrointestinal system.[1] In the mouse stomach, AQP4 is expressed mainly in the parietal cells[2] of the gastric glands of the corpus, at the level of the basolateral membranes. Lack of AQP4 in-KO-deficient mice does not seem to affect the secretion rates and composition of the gastric juice.[3] Anyway, little is known about the glycosylation patterns of the secreting cells, such as those involved in mucus secretion, which plays an important role in protecting the mucosa from lesions from gastric juice, mechanic injuries and pathogens. In this contribution, we characterize the secreting cell types in the gastric glands of AQP4-deficient-knockout (KO) mice in comparison to wild-type (WT) by morphological, lectin-histochemistry, and immunohistochemistry analyses. Sections, 5-µm thick, were analyzed by histochemical (PAS, AB pH 2.5), lectin binding (SBA, DBA, PNA, WGA, GSAII, Con A, UEA-I, AAA, LTA), and immunohistochemical (anti- β- H+/K+-ATPase, anti-pepsinogen) methods. Compared to WT mice, KO showed larger neck mucous cells with lower amounts of glycans with galactosyl/galactosaminylated, glycosyl/glycosaminylated and fucosylated residuals. Besides, in the KO a significantly larger number of parietal cells showed a reticular pattern, indicating a condition of active secretion. Thus, even if the gastric juice composition is unaffected, lack of AQP4 seems to affect morphological and glycosylation patterns as a possible consequence of altered conditions, that could lead to pathological consequences.

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MOVE OVER, OLD-SCHOOL ASSAYS: MEET THE REGULATORY VOLUME DECREASE (RVD) TEST, YOUR NEW BEST FRIEND FOR MEASURING CELLULAR DAMAGE!

F. Impellitteri[1], G. Piccione[1], C. Faggio[2]

[1]Department of Veterinary Sciences, and [2]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
E-mail: federica.impellitteri@studenti.unime.it

Environmental pollutants can cause significant damage to the cellular membrane, leading to the loss of important physiological functions. The commonly used cell viability assays such as Trypan Blue and Neutral Red assays are unable to detect these functional impairments, resulting in the underestimation of cellular damage. The Regulatory Volume Decrease (RVD) evaluation has been proposed to overcome this limitation as an additional, more sensitive, and reliable method for measuring cellular damage. Cells of osmoconform organisms, such as the cells of the mussel's digestive gland, can sense and respond to osmotic changes in their environment by adjusting volume. This involves the efflux of ions and osmolytes from the cell to regulate its volume and restore physiological functions. The RVD evaluation can detect changes in this process caused by pollutants, offering an accurate approach to evaluating cellular harm. To demonstrate the efficacy of this test, the study analysed the response of digestive gland cells of *Mytilus galloprovincialis* to the fungicide tebuconazole (TEB) at two concentrations, 2 ng/L (E1) and 2 µg/L (E2), as well as a control

group. The viability of the cells was assessed using Trypan Blue and Neutral Red assays, which did not reveal any significant differences between the control and exposure groups. However, the RVD test showed significant differences between the groups, exhibiting a reduced ability to regulate their volume compared to the control group. Overall, we can conclude that the RVD evaluation can be considered an additional tool for assessing the effects of environmental pollutants on cells due to its high accuracy and consistency in detecting cell damage. Indeed, the integration of the RVD assay alongside existing viability tests may provide a more comprehensive measurement of the functional capacity of cells.

EFFECT OF SUSTAINABLE PROTEIN SOURCES FROM *HERMETIA ILLUCENS* ON GROWTH PERFORMANCE OF ZEBRAFISH (*DANIO RERIO*) FROM LARVA TO ADULT

K. Riolo[1], C.F.C. Lanes[2], F.A. Pedron[2], S. Oliva[2], D. Savastano[3], A. Giannetto[1]

[1]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy; [2]Curso Superior de Tecnologia em Aquicultura, Campus Uruguaiana, Federal University of Pampa, Uruguaiana, Brazil; [3]Progetto Hermetia (www.progettohermetia.it)
E-mail: kririolo@unime.it

Hermetia illucens, commonly known Black Soldier Fly (BSF) is a saprophagous dipteran insect that has recently been attracting increasing interest as larvae represent valuable alternative protein sources that can be used in animal and aquafeed formulation. The present study investigated the effect of 100% fishmeal (control diet) replacement with defatted BSF meals obtained from two different developmental stage (V instar larvae and prepupae) on zebrafish (*Danio rerio*) growth performance and wellness. During the 60 days feeding trial, morphometric parameters (such as final total length, final standard length, final body weight, weight gain, daily growth rate and specific growth rate) and gene expression analysis of metabolic pathways such us muscle growth (*igf1, igf2, mstnb, myod1, myog, myf5*), hydrolysis of chitin (*chia.2, chia.3, chia.5*), stress (*hsp70* and *nr3c1*) and immune (*il1b, il6, tnfα*) responses were evaluated in zebrafish larvae (30 days post hatching, dph), juveniles (60 dph) and adult (90 dph). Results showed that both BSF-based diets do not negatively affect zebrafish performance. Noteworthy, the two BSF meals can differently modulate fish growth and the investigated metabolic pathways. Particularly, the different levels of chitin in the two BSF diets might have a specific impact on immune response by modulating *interleukins* and *tnfa* expression during zebrafish development with a peculiar response to the different BSF meals. The relevantly beneficial effect of prepupae meal on fish growth can be due to the significant activation of myogenic regulatory factors (MRFs), as assessed by gene expression analysis. Overall, these findings showed that both BSF meals can totally replace fishmeal in zebrafish diet without negative effects on fish growth performance and welfare and pave the way for the formulation of specific BSF diets for different fish developmental stages and species of economic interest in aquaculture.