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DOCTORATE (PH.D.)
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Candidate
Chandrashekar B.R

Tutor
Rita Carini

PROTECTION OF

STEATOTIC LIVER CELLS UPON

ACTIVATION OF

ADENOSINE A2a RECEPTOR

INDEX

INTRODUCTION

Non Alcoholic Fatty Liver Disease (NAFLD) and Non Alcoholic Steatohepatitis (NASH)

Nonalcoholic fatty liver disease (NAFLD) is characterized by accumulation of excess fat i.e when lipid in cell exceeds 5% of lipid of total liver weight (steatosis).The pathophysiological picture of NAFLD ranges from simple steatosis to lobular inflammation , parenchymal injury and to non alcoholic steatohepatitis (NASH) which can progress in fibrosis(1,2). They are considered as the hepatic manifestations of the so called 'Metabolic Syndrome' a cluster of closely related clinical features linked to visceral obesity that include insulin resistance, dyslipedemia and cardiovascular diseases. NAFLD/NASH is now the most frequent hepatic lesion in western countries with prevalence in the general population ranging from 3-15% but reaching up to 70% among overweight individuals (1,3). About 15- 20 % patients accounting for the pathological evolution form of NASH from the NAFLD with possible progression to cirrhosis or hepatocellular carcinoma(4).Several mechanisms, including oxidative stress, pro inflammatory cytokine production, unbalanced adipokine generation and mitochondrial dysfunction's, have been associated with the evolution of NAFLD to more severe liver injury(5,6). The primary factors for NAFLD progression to NASH is dysregulated lipid metabolism in liver and adipose tissue and also due to the increased insulin resistance(7,8). The circulating non esterified free fatty acids and their metabolites produced by denovo lipogenesis or due to over consumption of carbohydrates, can account to a phenomenon known as lipotoxicity and are regarded to play a role in NAFLD evolution to NASH(9,10). In particular non esterified fatty acid have been shown to apoptosis through the induction of endoplasmic reticulum stress and JNK activation(11,12).

The development of effective therapies with minimal side effects against NAFLD is vital for controlling the progression of this disease to advance to end stage liver disease. To this regard treatments targeting the JNKs may prove beneficial for preventing lipotoxicity and NAFLD progression to NASH since recent report suggests that pharmacological or genetic inhibition of JNK activation prevents lipotoxicity " in vitro" and improves the steatohepatitis condition in rodent models of NASH(14).

ISCHEMIC LIVER PRECONDITIONING:

Through extended ischemia-re-perfusion(IR)is deleterious to organs, it has been recognized since the 1980s that a short period of ischemia with subsequent re-perfusion triggers natural defense mechanism against future ischemic insults and protects the organ against the IR damage (IRI). This phenomenon, regarded as ischemic preconditioning(IP)(15). Was first observed in heart by Murray and et al in 1986 and later it was shown in other organs like liver(16,17). IP can be applied intermittently or as a single short period 5-10 ten minutes of ischemia followed by 10-15 minutes re-perfusion. The protection induced by IP takes place in two different phases. The first phase known as early preconditioning immediately follows the preconditioning stimulus and modulates different cellular functions . The second phase is known as delayed or late preconditioning; it starts 12-24 hours after the preconditioning stimulus, can last up to 3-4 days, and is characterized by gene transcription and " de novo" protein synthesis(18). Despite these differences, both phases of preconditioning can be initiate by the same stimuli and partially share the same intracellular signal pathways.

" In vivo" and "in vitro" studies have clearly established that the onset of IP is triggered by the production of adenosine and by the subsequent stimulation of adenosine A2a receptor (18,19-22). This was confirmed in our laboratory with experiments using primary rat hepatocytes preconditioned with10 mins of hypoxia plus 10 mins of re-oxygenation. In this model , the released adenosine to extra-cellular induced hepatocyte protection by the autocrine stimulation of A2A receptors. Ischemic preconditioning has also shown to be effective in reducing re-perfusion damage during hepatic resection in humans(23), as well as to improve the outcome of hepatic transplants in experimental animals. These beneficial effects are particularly evident in fatty livers where preconditioning almost reduced 50% , the release of transaminase level and an histological evidence of necrosis(24).

The ischemia re-perfusion injury is regarded as a major cause of liver dysfunction or failure , after tissue resection and transplantation. Such problems are particularly evident in patents with fatty livers that are more susceptible to IRI and that are now often employed as liver donors because of the shortage of organs.

The discovery surgical ischemic preconditioning raised hopes that it could be applied to patients to prevent the side-effect of major liver surgery. The first application of IP in clinical trials, however, has given conflicting results. In some cases, in fact , IP did not afford protection and in some others its protective effects were extremely variable(25,26-30). This indicated that a different and more reliable approach was needed to activate in patients the intrinsic protection mechanisms.

ADENOSINE AND ADENOSINE RECEPTORS

Adenosine is an endogenous purine nucleoside that modulates many physiological processes. Extracellular adenosine concentrations in normal cells are approximately 300 nM but this concentrations are elevated quickly during tissue damage and inflammatory reactions .

The released adenosine interacts with different subtype of adenosine receptors that modulate cell protection , inflammation and immunological responses. There are four kinds of adenosine receptors A1, A2A, A2B and A3 which are of purinergic class and G protein coupled receptor. Interestingly, some findings indicate that the different adenosine receptors might have dissimilar or even opposite effects. Well characterized is the pro- inflammatory activity of A1 and A2b receptor and, in contrast, the immune -suppressive action of the A2a receptor. Stimulating are also different effects of adenosine receptors on liver steatosis and lipotoxicity. In A2ab KO mice ethanol -induced hepatic steatosis is reduced compared with WT mice.(31) indicating a pro-steatotic action of A2b receptor. On the other hand, recent studies in our laboratory have shown the protective effect of A2aR stimulation from lipoapoptosis(14).

RATIONALE OF THE PROJECT:

Aim of my this project is to investigate the hypothesis that surgical ischemic preconditioning (IP) does not produce reliable results against I/R injury of steatotic liver because its main trigger, adenosine, might induce conflicting (i.e.hepatotoxic/hepatoprotective) effects by activating the different adenosine receptors.

In particular, I will examine the possibility that pharmacological activation of selective adenosine receptors is more or less effective that IP or adenosine treatment in affording protection of steatotic liver cells and of fatty liver exposed to I/R.

The results obtained would offer the rational base to propose that pharmacological activation of a specific adenosine receptor is a more efficient and reliable procedure to activate the intrinsic system of hepatoprotection that IP itself.

Based on this General Aim, in the first months of my PhD, I have preliminarily investigated the pro/anti-steatotic and pro/anti-lipotoxic actions of adenosine, A2aR , A2bR or A1R agonist in the mice liver cell line C1C7 exposed to the free fatty acid Palmitic acid (PA).

MATERIALS AND METHODS:

MATERIALS:

The chemical substances used were obtained from the following company-Minimum essential medium Eagle (DMEM M4655) , Non essential Amino-acid, Vitamin solution, Sodium Pyruvate , Dimethyl Sulfoxide (DMSO D8418), Trypsin-EDTA (T3924) Phosphate Buffered saline (P4417), MTT Assay compound Thiazolyl Blue Tetrazolium Bromide (M2128) , Trypan blue (T8154) ,Palmitic acid, CGS-21680 (C-141), Adenosine minimum 99%, CPDX and CCPA were brought from Sigma aldrich for the experimental use. The antiobiotics Penicillin/Streptomycin were obtained from PAA laboratories Germany. The Foetal Bovine Serum (FBS) was obtained from GIBCO company. The Steatosis colorimetric assay kit was brought from CAYMAN company. BAY 60-6583 , MRS – 1706 and ZM -241385 were brought from Tocris Bioscience .

METHODS:

C1C7 culture and treatments:

The HEPA-1 wild type C1C7 Hepatocarcinoma cell line was obtained from the European Collection of Cell Cultures and cultured in modified DMEM medium containing 10% FBS (fetal bovine serum), 5% penicillin/streptomycin, 100X Nonessential amino acid, 1% vitamin solution and 1% sodium pyruvate. For Cell viability and Steatosis assay the C1C7 cells were treated with fresh medium supplemented with Palmitic acid (700uM: sigma) for 16 hours to induce steatosis in cells. CGS 21680(**5 uM/ml**), CCPA (**100 uM/ml**), BAY 60-6583 (**3 nM)** were used as agonists of A2A, A1and A2B receptors and CPDX-(**100 uM**), MRS 1706- (10 mM/ml) and ZM241385 (**1 uM/**) as antagonists of A1, A2B and A2A receptors respectively along with adenosine(**250 uM)**. Chloroquine (25uM) was used as a positive control in steatosis assay. Cell viability was evaluated by Trypan blue test and MTT assay while the intracellular lipid concentration using steatosis colorimetric assay according to manufacturers instructions .

DETERMINATION OF CELL VIABILITY:

1) Trypan Blue Test

Trypan Blue Solution, 0.4%, is routinely used as a cell stain to assess cell viability using the dye exclusion test. This test is often performed while counting cells with the Burker slide during routine sub-culturing. The dye exclusion test is based upon the concept that viable cells do not take up Trypan Blue, because the cell membrane will be intact but in dead cells the cell membrane are ruptured and are permeable take up the dye this is the basic principle for differentiating viable and dead cells.

Procedure for Trypan blue test- Centrifugation of cell suspension is done , later the pellet obtained is retained after discarding the supernatant. The obtained pellet is resuspended in PBS or serum free medium. Later one part of cell suspension and 9 parts of Trypan blue mixed and cell count is done. A drop of Trypan blue and cell cell mixture is added to Burker slide and counted under microscope for unstained viable cells and stained non viable cells.

2) MTT ASSAY:

MTT assay is a colorimetric assay used to measure the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT which is added to the medium is taken up by the cell passes on to the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. Later the cells are solubilised with an organic solvent (eg. DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can take place only in metabolically active cells the level of activity (the colored product)is direct measure for viability of cells .

Procedure for MTT assay – Prepared MTT solution from (5mg/ml in PBS 1X)which 100 ul is added to each well containing 1ml of medium where cells treated and later incubated for 30 minutes in the incubator at 37 degrees. After medium is removed and 500uL of DMSO are added . Plate covered with a box to create dark area is kept on basiculant for 15 minutes . Then the solution with cells are mixed well with the pipette before reading the plate in the spectrophotometer at 570 nm.

DETERMINATION OF STEATOSIS:

Steatosis colorimetric Assay : To evaluate the intracellular lipid concentration we used the steatosis colorimetric assay. This assay provides a convenient tool for evaluating steatosis where the neutral lipids are stained using oil red O stain .After the staining procedure done as indicated in the data sheet kit, we quantified the lipid accumulation with the dye extract solution where dye is extracted from the lipid droplets is quantified using the plate reader in spectrophotometer at 490 nm.

Procedure of Steatosis Assay:- Medium is removed from **t**reatment finished cells and washed nicely with the PBS 1x twice. Later the cells are fixed to the plate with 1x fixative agent to all wells. Later the wells are subjected to washing with wash solution for 5 minutes and kept on the basculant for 5 minutes . Later second wash is given before the plate is air dried completely . Once the wells are dry, each well is treated with the oil red solution and incubated for 20 minutes. Later the oil red solution is removed and the wells are washed with distilled water some times until the wash solution contains no visible pink color. Then wells are washed twice for 5 minutes each with wash solution and completely air dried for 20 minutes. For quantification of lipid accumulation inside the cells , a 100 uL/well lipid droplets assay dye extraction solution is added and incubated for 30 minutes on the basculant and the absorbance read in spectrophotometer at 490nm.

RESULTS:

EFFECT OF ADENOSINE RECEPTORS STIMULATION ON LIVER CELLS STEATOSIS INDUCED BY PALMITIC ACID

Recent results obtained in our Laboratory demonstrated that the circulating free fatty acid Stearic Acid (SA) was able to induce steatosis and lipotoxic effects in the rat hepatoma cell lines HTC and in primary rat hepatocytes (14).

In the present project I have set up a further model of steatosis and lipotoxicity by employing the saturated free fatty acid Palmitic acid (PA) and the mice hepatoma cell line, C1C7. PA was here employed, since it is regarded as more clinically relevant than SA, being found more toxic and also present in higher concentration then SA in the serum of NASH patients.

In preliminary experiments I evaluated the prosteatotic and lipotoxic effects of PA 250, 500, 700 and 1000 μM at different times of treatment: 8, 16 and 24 hours (not shown). I found that the concentration of 700 μM and the treatment of 16 h, represented the lower concentration and the lower time of treatment to produce pro-steatotic and lipotoxic effect. These experimental conditions were therefore chosen for the subsequent determinations.

Figure 1 illustrates the increase of lipid content of C1C7 cells after PA treatment, with PA given at 700uM final concentration for 16 hours. Intracellular lipid accumulation was evaluated using a steatosis colorimetric assay with spectrophotometer quantification (see Methods) .

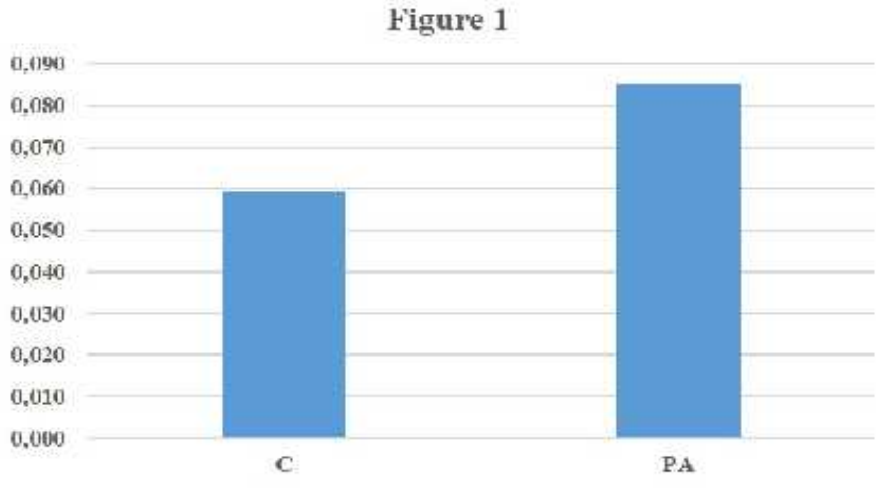

Figure 1: Palmitic acid treatment induces steatosis

The role of adenosine and of the agonists of adenosine receptors A2a, A2b and A1 in modulating lipid accumulation was then evaluated. To this purpose cells were treated with PA(700uM) in presence or in absence of adenosine (250 μM) or of the A2aR agonist CGS21680 (5μM), of the A1R agonist BAY (3nM) or of the A2b agonist CCPA (100 μM).

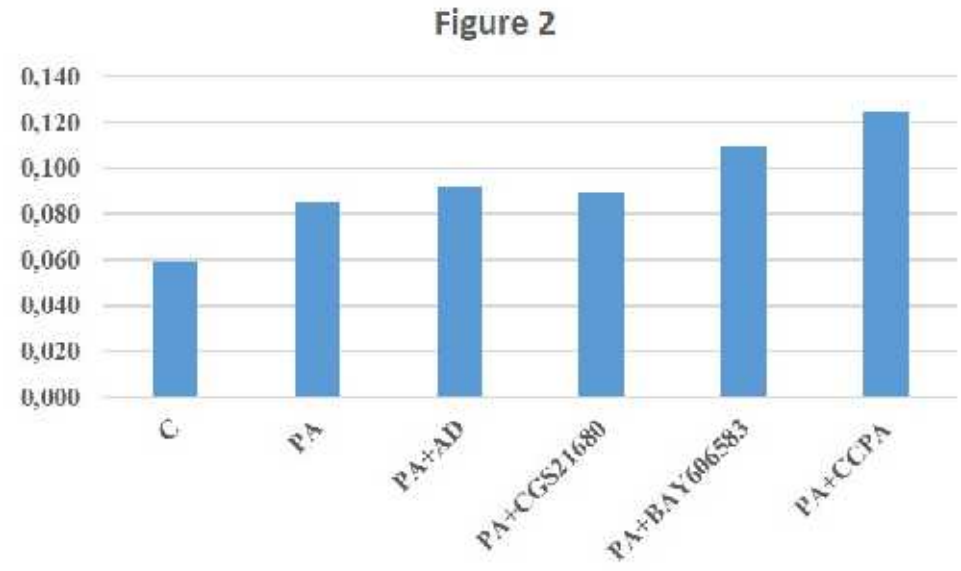

Figure 2: Lipid content of C1C7 cells exposed to PA in presence or in absence of adenosine or different receptors agonist

As shown in figure 2, adenosine treatment did not modulate steatosis compared to PA alone. The same effect was produced by CGS21680 the agonist of A2a receptors. In the case of PA plus BAY 606583 the A2B receptor agonist, there was an increase of intracellular lipid accumulation respect to PA alone. Such lipid increase was more marked in the last condition with PA plus CCPA the A1 receptor agonist .

EFFECT OF ADENOSINE RECEPTORS STIMULATION ON LIVER CELLS CYTOTOXICITY INDUCED BY PALMITIC ACID

Figure 3 shows the decrease of cell viability of C1C7 cells, as evaluated by MTT assay, after treatment for 16 hours with Palmitic acid at 700uM final concentration.

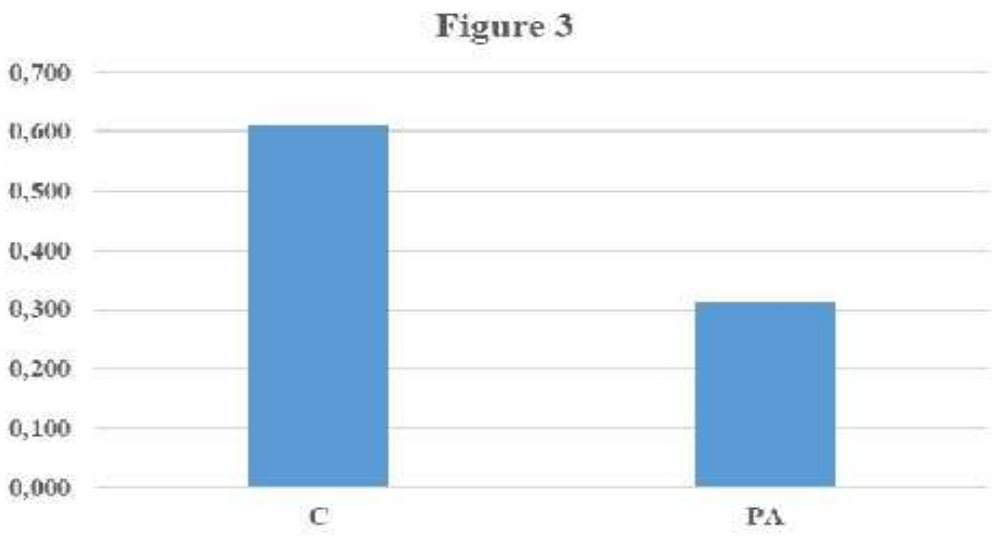

Figure 3:

Lipotoxic effect of Palmitic acid in C1C7 cells

Given the association from the prosteatotic action of PA (Fig. 1) and its cytotoxic activity (Fig. 3) we evaluated the effect of the treatment with adenosine and of the stimulation of different adenosine receptors that I previously observed to modulate cells steatosis after PA treatment (Fig. 2).

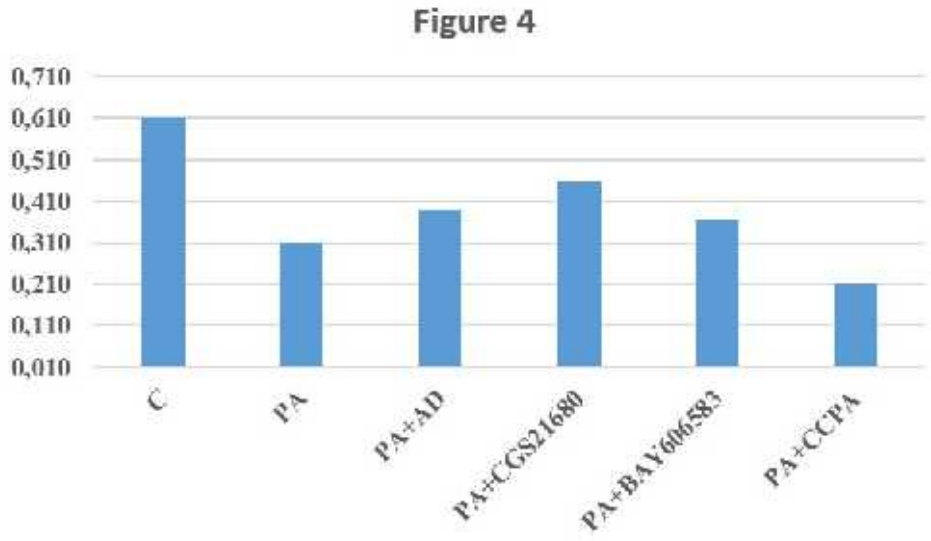

Figure 4: Effect on viability of PA-treated cells of adenosine and of the different adenosine receptors agonist

From the figure 4 it is evident that after adenosine treatment there is a slight increase in the cell viability respect to PA alone .The next condition is Palmitic acid plus CGS21680 that is the agonist of A2a receptor. As we can see in fig 4, CGS21680 reduced the loss of viability induced by PA. The next condition is PA plus BAY 606583 that is A2B receptor agonist. The viability of cells treated with BAY was similar to those treated with PA alone. The last condition was PA plus CCPA, the A1 receptor agonist . CCPA increased the loss of viability induced by PA.

DISCUSSION:

In this study I evaluated the effects of adenosine and of pharmacological activation of A1, A2a and A2b adenosine receptors with their specific agonists and after palmitic acid treatment on steatosis and viability of mice hepatoma C1C7 cells.

At first I evaluated the pro-steatotic effect of Palmitic acid (PA), a saturated free fatty acid. PA effectively induced steatosis compared to control. Later we checked if the steatosis was modulated by specific agonists of the adenosine receptors like CGS21680 that is the agonist of the A2aR receptors, BAY 606583 that is A2B receptor agonist and CCPA that is A1 receptor agonist. As shown in the results, CGS21680 did not modulate the steatotic action of PA, whereas BAY606583 and, more conspicuously, CCPA increased the intracellular lipid accumulation more than palmitic acid alone.

It was previously shown that in NAFLD disease, hepatocytes incapability to esterify the excess of NEFAs (non-esterifed “free” fatty acids) triggers apoptosis. I thus established an “in vitro” model of mice liver cells lipotoxicity, investigating if Palmitic Acid was cytotoxic for C1C7 cells line. My results showed that the cell viability after palmitic acid treatment decreases respect to the control. I also observed, the A2a receptor agonist CGS21680 was able to prevent such loss of viability whereas the other agonists of adenosine receptors were not effective or even more toxic than PA alone. In particular, I found that the A1R agonist, that markedly enhanced liver cells steatosis compared with PA alone, also clearly enhanced PA-induced lipotoxicity suggesting a correlation between lipid accumulation and lipotoxicity. As regard the effect of the treatment with adenosine alone, adenosine protected cells from damage but its effect was lower than CGS216780.

These first and preliminary results, suggest that the activation of A2a receptors might be more effective in preventing lipotoxicity than adenosine, since adenosine by activating all its receptors, also activates A1 receptors that, by promoting an higher increase of cellular lipids, increases lipotoxicity of PA. Such observations encourages future investigations aimed to demonstrate that pharmacological activation of A2a receptor might represent a more efficient technique that IP application in preventing steatotic liver cells toxicity and fatty liver damage after I/R.

FUTURE PROSPECTS:

In “vitro studies”:

1) Demonstrate that pro-steatotic action of A1 receptor is responsible for the lower protective effect of adenosine compared to CGS21680 by using antagonist of A1 receptor in addition to adenosine.
2) Evaluate the cyto-protective activity of CGS21680 in comparison with adenosine and A1 receptor agonists in steatotic hepatocytes exposed to hypoxia-re-oxygenation (to simulate “in vitro” the I/R of steatotic liver).
3) Investigate the molecular mechanisms responsible for the cytoprotective action of CGS21680 during hypoxia-re-oxygenation of Steatotic hepatocytes (with particular regard to the possible protective role of JNK activation against the mitochondrial alterations and the possible effects on ER stress)

In “vivo studies”:

1) Develop a model of mice IRI of Steatotic liver
2) Evaluate and compare the effect of “in vivo” treatments with IP, adenosine, CGS21680 or CCPA on the IRI of Steatotic liver.

PARTICIPATION TO ADDITIONAL EXPERIMENTAL ACTIVITIES

My Project is part of the General Project "Pharmacological and genetic activation of A2a Receptor in Steatotic and non steatotic liver". During the first months of my PhD I also participated to several experimental activities related to this General Project.

In particular, I took part to the preparation of primary hepatocytes, endothelial and Kupffer cells isolated from mice liver exposed to I/R obtained from animals treated or not with A2aR agonists. These cells were then employed for Proteomic analysis finalized to evaluate the pattern of protein expression of parenchymal and not parenchymal mice liver cells during IRI and treatment with A2aR agonists.

I also participated to experiments aimed to induce NASH in mice fed with MCD diet treated or not with A2aR agonists and to the determination of the different parameters of liver damage. These experiments were finalized to evaluate the different lymphocyte populations activated in mice during NASH development and the modulatory effect of the treatment with A2a agonists.

I finally participated to the breeding and expansion of a population of A2aR KO mice to be employed in future experiments with chimeric A2aR mice.

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CONGRESS/CONFERENCES ATTENDED

1) EUROPEAN ASSOCIATION FOR THE STUDY OF LIVER 2013 – Amsterdam, 24-28 April 2013

2)WORKSHOP AUTOANTIBODIES – Novara, 21-22 March 20

SEMINARS ATTENDED

1) "Alumina/Zirconia composites for hip joint applications"–Dr.Alessandro Alan Porporati–6 Manager of Scientific Affairs, Medical Products Division, Ceramtec GmbH, Italy

2)"Long non-coding RNAs and a typical protein-coding genes as new players in the regulation of neuronal functions" –Dr. Silvia Zucchelli –neo RTD BIO18 (genetics) of the Department of Health Sciences, Italy

3) "Glioblastoma stem cell biology and its implications in cancer therapy" – Dr. Giuliana Pelicci – Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

4)"Carbapenemases: a last frontier for beta-lactam antibiotics?"–Prof. Giuseppe Cornaglia– Dipartimento di Patologia e Diagnostica Università degli studi di Verona, Italy

5) "Red blood cells as carriers for magnetically targeted delivery of drugs" –Prof. Dr. Hans Bäumler –Berlin-Brandenburg Center for Regenerative Therapies, Charité – Universitätsmedizin Berlin

6) " Skin cancer in vivo models, what they have and can tell us" –Dott. Girish Patel – School of Medicine, Cardiff University, United Kingdom

7) " Stem/progenitor cell transplantation in the rat: A powerful tool to study tissue replacement in the normal and diseased liver" –Michael Oertel– School of Medicine,Dept. of Pathology, University of Pittsburgh (USA)

8) Cytotoxic potential of plasmacytoid dendritic cells in autoimmune diseases - Prof. Silvano SOZZANI,Department of Molecular and Translational Medicine, University of Brescia

9) Interleukin -33: a novel player in chronic intestinal inflammation. Dr. Luca Pastorelli Department of Biomedical Sciences for Health, IRCCS Policlinico San Donato San Raffaele del Monte Tabor Foundation, Milan.

10).Autophagy and human disease; model Cystic Fibrosis. Prof. Luigi Maiuri, University of Foggia, European Institute for Research in Cystic Fibrosis, Division of Genetics and Cell Biology, IRCCS S. Raffaele Milan.

11).Mechanisms of chronic inflammation and immunoregulation in infections and tumors. Speaker - Vincenzo Barnaba, La Sapienza University of Rome.