Laboratory Exercise

Oxidative Damage in Diabetics: Insights from a Graduate Study in La Reunion University

From the Groupe d’étude sur l’inflammation chronique et l’obésité (GEICO), Plateforme CYROI, Université de La Réunion, Saint Denis de La Réunion, France

Abstract

Due to the growing incidence of diabetes in developed nations, there is a compelling case to be made for teaching graduate students more deeply about mechanisms underlying this disease. Diabetes is associated with enhanced oxidative stress and protein glycation via the covalent binding of glucose molecules. Albumin represents the major plasmatic protein and undergoes enhanced glycoxidative modifications in diabetic condition. La Réunion Island, a French department located in the Indian Ocean exhibit a growing incidence of diabetes. At the University of La Réunion, our research group named GEICO (Groupe d’Etude sur l’Inflammation Chronique et l’Obésité) participated to foster research and training in diabetes context and focuses on the impact of glycated albumin mediated oxidative stress on cell physiopathology. A laboratory course was designed by our group to introduce graduate students to cutting edge techniques in redox biology while providing insights into scientific processes and methods. This two weeks research laboratory training took place at CYROI, a local biotechnology center that provides advanced facilities for research, business, and education. Using histochemistry, molecular biology, biochemical techniques, student investigated oxidative damages in liver from leptin receptor deficient diabetic mice compared to control littermates. In addition, they used an in vitro model by assaying oxidative impact of glycated albumin on hepatoma carcinoma HepG2 cells. This article gives an overview of the organization and protocol used by the students during their two weeks training in the laboratory. Therefore, it may be helpful for teaching graduate students techniques used in research laboratory working on redox biology. © 2014 by The International Union of Biochemistry and Molecular Biology, 00(0):000–000, 2014.

Keywords: diabetes; oxidative stress; oxidation; University of La Réunion; glycated albumin

Introduction

Free radicals and reactive oxygen species (ROS) can induce oxidative stress and oxidative damages associated with the progression of age-related diseases [1]. Diabetes is characterized by hyperglycemia and insulin resistance and leads to dramatic complications such as cardiovascular disease, which remains the leading cause of mortality in western countries [2]. Diabetic complications arise as a result of oxidative stress due to the increased formation of ROS as well as a decrease in the antioxidant defence system. Oxidative stress can be promoted by hyperglycemia through the formation of advanced glycation end products (AGEs) [3]. Diabetes is a worldwide clinical disorder whereby it has been predicted that deaths from this chronic disease will double between 2000 and 2030 [4]. In the Indian ocean, the inhabitants of La Reunion Island, a French overseas department, exhibit high prevalence of type 2 diabetes (17.7% for men and 17.3% for women) [5].

Abbreviations: ROS, reactive oxygen species; AGEs, advanced glycation end products; GEICO, Grouped’Etude sur l’Inflammation Chronique et l’Obésité (obesity and chronic inflammation research group); CYROI, cyclotron region ocean indien (Cyclotron Indian ocean area); B4, Biochimie, BiologieMoléculaire, Biomédicine et Biotechnologies (Biochemistry Molecular Biology, biomedicine and biotechnology); FACS, flow cytometry and fluorescence activated cell sorting; PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substances quantification

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At the University of La Réunion, our research group named GEICO (“Groupe d’Etude sur l’Inflammation Chronique et l’Obésité” or obesity and chronic inflammation research group) participated to foster research and training in diabetes context and focused on the impact of glycated albumin mediated oxidative stress on cell physiopathology [6–8].

In 2013, a laboratory course was designed by our group to teach graduate students cutting edge techniques in redox biology. This two weeks laboratory training took place at CYROI (“cyclotron region ocean indien” or Cyclotron Indian ocean area), a local biotechnology center that provides advanced facilities for research, business, and education. Using histochemistry, molecular biology, and biochemical techniques, student investigated hepatic oxidative damages in leptin receptor deficient diabetic mice (Lepr<sup>db</sup>, Db/Db transgenic mice) and compared with control littermates. In addition, they used an in vitro model by assaying oxidative impact of glycated albumin on hepatoma carcinoma cells (HepG2 cells).

The laboratory training was addressed to students in first year master degree entitled B4 for “Biochimie, Biologie Moléculaire, Biomédecine et Biotechnologies” (Biochemistry, Molecular Biology, Biomedicine, and Biotechnology).

The laboratory course started with a one-hour lecture session to acquaint the students with the objectives, methods, timetable (Table 1), and for an introduction to safety rules.

Pre-laboratory Preparation
We First Acquainted Students with Background and Overview on Oxidative Stress
Free radicals, ROS, nitrogen, and chlorine species contribute to the development of age-related diseases through oxidative stress and oxidative damage induction. Oxidative stress results from a disruption in the prooxidant and antioxidant balance and induces damages to organic compounds such as lipids, proteins, and nucleic acids [9]. So, either a reduction or antioxidant levels or/and an increase in ROS lead to oxidative stress [10]. Enhanced oxidative stress was evidenced in diabetes pathology [3]. Oxidative stress in diabetes may result from several processes such as elevated ROS production from glucose autoxidation, glycoxidized proteins, and impairment of antioxidant proteins [11].

Explanation of AGEs Formation
Figure 1 corresponds to the slide used to explain AGE formation and impact on cells. Diabetes is associated with enhanced oxidative modifications of serum proteins such as albumin. Non-enzymatic glycation of proteins, leading to the formation of AGEs, is enhanced in diabetic patients and the percentage of glycated hemoglobin HbA1c is commonly used as a clinical indicator to monitor the development of hyperglycemia [6]. In chronic hyperglycemic conditions, albumin undergoes increased glycation and glycoxidation [12]. The glycation phenomenon corresponds to the non-enzymatic and non-oxidative covalent binding of glucose to a protein [12]. Glycoxidation corresponds to the radical-mediated oxidation reaction of both free and protein-bound sugars [13]. Amadori rearrangement of glycated protein leads to the formation of advanced glycoxidation (also termed advanced glycation) end products (AGEs) [14, 15]. AGEs accumulate in the tissues of diabetic patients and contribute to the disease progression [3]. Indeed, several studies report that glycoxidative modification of proteins exerts a pivotal and causative role in the pathogenesis of diabetes [1, 16–18]. Binding of AGEs to their receptors (RAGE) triggers several cellular phenomena potentially relating to diabetic complications. AGEs were demonstrated to enhance inflammation in monocytes via RAGE-mediated pathways and leading to vascular cell dysfunction [19, 20].

We Took Advantage of this Laboratory Training to Describe Research Objectives of Our Group at La Reunion
Researches performed in our group focus on the effects of oxidative modifications induced by the glycation of albumin on its structure, antioxidant properties, and on cell physiology [6–8, 21–27]. The implication of glycoxidized albumin in the generation of oxidative stress and damages on cells still needs to be better specified.

Organization of master laboratory training relies on research groups from the University of La Réunion. This year, our GEICO team prepared a research laboratory course on the oxidative stress/cell physiopathology interaction theme according to our group expertise.

We noticed students are very motivated to participate to a laboratory practical training which resembles more to real research rather than a “classical” academic approach. We did our best to make them realize they are co-researchers whose results are important rather than just students who need to learn a technique.

Objectives of the Laboratory Training were Explained to Students as Objective of a Research Program which Must Lead to a Scientific Publication
Objectives of the laboratory training were to assess liver oxidative damages in diabetes by the use of different methods such as molecular biology and biochemical techniques, cellular, and ex vivo approaches. More specifically, oxidative damages were evaluated in liver samples from diabetic Db/Db or non-diabetic control transgenic mice and in HepG2 cell line incubated with native or glycated albumin.

Methodologies Taught and Used During the Laboratory
Students were given a laboratory protocol book (document no. 1) containing all protocols used. They included:
### Table 1: Time table for the two weeks laboratory

#### Week 1:

<table>
<thead>
<tr>
<th>Day</th>
<th>Date</th>
<th>Time</th>
<th>Activity</th>
<th>Participants</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>Monday, 10/7/13</td>
<td>A.M.</td>
<td><strong>TP presentation and GLP Training</strong></td>
<td>G1 &amp; G2</td>
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<tr>
<td></td>
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<td><strong>RNA extraction from liver tissues in Trizol</strong></td>
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<td></td>
<td></td>
<td>P.M.</td>
<td><strong>RNA quantification and control (agarose gel)</strong></td>
<td>G1 &amp; G2</td>
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<tr>
<td><strong>Day 2</strong></td>
<td>Tuesday 10/8/13</td>
<td>A.M.</td>
<td><strong>qPCR: GAPDH and catalase genes expression</strong></td>
<td>G1 &amp; G2</td>
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<td><strong>Protein extraction from liver tissues</strong></td>
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<td></td>
<td>P.M.</td>
<td><strong>Catalase activity</strong></td>
<td>G1 &amp; G2</td>
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<td><strong>Protein quantification by BCA method</strong></td>
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<tr>
<td><strong>Day 3</strong></td>
<td>Wednesday 10/9/13</td>
<td>A.M.</td>
<td><strong>Cell Culture: AGE stimulation of HepG2 cell lines (RNA) for 4H.</strong></td>
<td>G1 &amp; G2</td>
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<td><strong>Agarose gel electrophoresis for GAPDH and catalase PCR amplicons</strong></td>
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<td><strong>AGE characterization: fructosamine assay</strong></td>
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<td></td>
<td>P.M.</td>
<td><strong>Cell Culture: Collect treated HepG2 cells in trizol + supernatant collection</strong></td>
<td>G1 &amp; G2</td>
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<td><strong>Course: Use of main software (Endnote, Prism)</strong></td>
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<td><strong>How to write an Article and perform statistics</strong></td>
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<td><strong>Day 4</strong></td>
<td>Thursday 10/10/13</td>
<td>A.M.</td>
<td><strong>Cell Culture: Subculture of HepG2 cell lines (cellular culture)</strong></td>
<td>G1</td>
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<td><strong>TBARS assay on liver tissues</strong></td>
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<td><strong>q PCR results analysis</strong></td>
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<td>P.M.</td>
<td><strong>Mouse dissection and organs harvesting</strong></td>
<td>G2</td>
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<td><strong>Immunohistology: Liver tissues preparation, staining and microscopy visualisation</strong></td>
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<tr>
<td><strong>Day 5</strong></td>
<td>Friday 10/11/13</td>
<td>A.M.</td>
<td><strong>Mouse dissection and organs harvesting</strong></td>
<td>G1</td>
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<td><strong>Immunohistology: Liver tissues preparation, staining and microscopy visualisation</strong></td>
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<td>P.M.</td>
<td><strong>Cell Culture: Subculture of HepG2 cell lines (cellular culture)</strong></td>
<td>G2</td>
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<td><strong>TTBARS assay on liver tissues</strong></td>
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<td><strong>q PCR results analysis</strong></td>
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#### Week 2:

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<th>Date</th>
<th>Time</th>
<th>Activity</th>
<th>Participants</th>
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<tbody>
<tr>
<td><strong>Day 6</strong></td>
<td>Monday, 10/14/13</td>
<td>A.M.</td>
<td><strong>Cell Culture: AGE stimulation of HepG2 cell lines (protein) for 24H.</strong></td>
<td>G1 &amp; G2</td>
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<td>P.M.</td>
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</table>
Molecular biology techniques such as RNA extraction quantification and quality control assessment, reverse transcriptase (RT), real time polymerase chain reaction (qPCR).

Biochemistry protocols including catalase activity measurements, thiobarbituric acid reactive substances quantification (TBARS), fructosamine assay, electrophoresis and Western-blotting, ELISA. (cf. Fig. 2).

Cellular approaches were detailed, including preparation of tissue culture medium, HepG2 cells subculture, stimulation of HepG2 cells with HSA and HSA-AGE, cell counting, flow cytometry, and fluorescence activated cell sorting (FACS).

Ex vivo studies concerning animal dissection, immunohistochemistry, mouse liver homogenisation, analyses on homogenates.

Scientific Report
Explanations were provided to students regarding the scientific report they were tasked to write from data obtained...
Overview of the methodologies used during the training. Objectives of the laboratory was to assess liver oxidative damages in diabetes by the use of different methods such as molecular biology and biochemical techniques, cellular and ex vivo approaches. More specifically, oxidative damages were evaluated in liver tissues from diabetic Db/Dk or normoglycemic control transgenic mice and in HepG2 cell line incubated with native or glycated albumin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIG 1**
Formation of AGEs. The glycation phenomenon corresponds to the non-enzymatic and non-oxidative covalent binding of glucose to a protein. Amadori rearrangement of glycated protein leads to the formation of AGEs (also termed advanced glycation). Binding of AGEs to their receptors (RAGE) triggers several cellular phenomena potentially relating to diabetic complications. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIG 2**
Overview of the methodologies used during the training. Objectives of the laboratory was to assess liver oxidative damages in diabetes by the use of different methods such as molecular biology and biochemical techniques, cellular and ex vivo approaches. More specifically, oxidative damages were evaluated in liver tissues from diabetic Db/Dk or normoglycemic control transgenic mice and in HepG2 cell line incubated with native or glycated albumin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIG 3**
Histological analysis of liver morphology and lipid accumulation. Paraffin or cryosections of liver (5 µm width) from Db/+ or Db/Db mice were stained with eosin hematoxylin, lipid droplets were stained using O red oil. Images were taken using a 32x magnifier. (cf. Protocol no. 16: Mouse liver Hematoxylin & Eosin, Oil Red O and b-actin page 29 of the laboratory protocol booklet). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
from the whole group (15 students). After the two-week laboratory course, each student had 15 days to provide a report in a scientific paper format (either in French or English language).

Safety Issues
Students were introduced to all our laboratory rules by Dr. Pierre Giraud, engineer in our lab. A reminder of the good use of personal protective equipment such as eye protection, lab coats, shoes, and gloves was made as well as caution in moving hazardous materials between laboratories. Special emphasis was also placed on waste collection and disposal (correct containers).

Results and Discussion

Representative Data Obtained by the Students
It was important for the students to assess the physiological impact of diabetes. For this purpose, we made students work on liver tissue from both diabetic mice (Db/Db) and their normoglycemic controls (Db/+). They had information about their biological parameters in terms of body weight and fasting glycemia (data sheet given during prelab setting). They first experienced the physiological aspect of the liver samples in term of color as steatosis was evident in Db/Db mice. By using histochemistry they evidenced more lipid accumulation in liver issued from Db/Db mice than in control mice (Fig. 3).

During the course, students had a 4-hr training about how to handle main software used in research, how to write an article, and perform statistics (cf. Table 1).

Catalase activity measured in liver from Db/+ or Db/Db mice. Protocol used to measure catalase activity is detailed on page 18 of the laboratory protocol booklet. (NS not significant; n = 5).

Characterization of fructosamine content in glycated albumin preparations. Fructosamine content in HSA and glycated HSA (AGES) preparation was determined using NBT reagent (cf. protocol no. 10: Fructosamine assay with NBT page 20 of laboratory protocol booklet). **p < 0.001 (vs. HSA) by Student’s t test (n = 7).

Catalase expression measured in liver from Db/+ or Db/Db mice. mRNA encoding catalase were measured by qPCR and normalized using GAPDH gene expression (cf. Protocol no. 6: Real time Polymerase Chain Reaction page 12 of laboratory protocol booklet). (NS not significant; n = 5).

Catalase activity in HepG2 cells treated with native or glycated albumin (AGE). HepG2 cells were incubated in the absence (PBS) or presence of 20 μM native (HAS) or glycated albumin (AGE) for 24 hr. Protocol used to measure catalase activity is detailed on page 18 of the laboratory protocol booklet. *p < 0.05 (vs. PBS) by Student’s t test (n = 6).
Students performed many biochemical analyses on liver tissue homogenates. For example, student performed catalase activity and expression measurements (Figs. 4 and 5). They experienced high heterogeneity in their results as no statistically significant variation was observed.

We made student characterized their albumin preparations (native and glycated albumin) in terms of fructoseamine content (Fig. 6). Students were taught fructoseamine, known as "early glycation adducts," could subsequently give rise to irreversible conjugates, called AGES. They were informed that fructoseamine levels can be used to monitor hyperglycemia in diabetes patients.

Students also measured catalase activity and expression in hepatoma HepG2 cells treated in the absence or presence of native or glycated albumin (Figs. 7 and 8). They evidenced significant increases in both catalase activity and expression for AGE-stressed cell in comparison with control cells (PBS). They could appreciate the reduced variability in their results when working on cellular lysates compared to tissue homogenates.

By using FACS technique, students evaluated several parameters in their treated HepG2 cells such as intracellular free radical formation and cellular viability (cf. protocol no. 19: Lab booklet). Student evidenced a significant impairment in cell viability when treated with AGEs in comparison with cells incubated with native albumin (Fig. 9).

By using Western blot technique and antibodies directed against oxidized protein, student evidenced a higher oxidation status of proteins when issued from Db/Db mice or from AGE-treated cells in comparison with control mice or native albumin-treated cells (Fig. 10).

**Student Evaluation**

The two-week laboratory research training ended with a 2 hr exam containing 20 questions relative to all parts of the laboratory. Here are few examples:

- What are the main basic safety rules in a research laboratory (6 minimum)?
- Give one advantage and one a disadvantage in using paraffin sections versus cryosections.

![FIG 8](image)

*Catalase expression in HepG2 cells treated with native or glycated albumin (AGE). HepG2 cells were incubated in the absence (PBS) or presence of 20 μM native (HAS) or glycated albumin (AGE) for 24 hr. mRNA encoding catalase were measured by qPCR and normalized using GAPDH gene expression (cf. Protocol no. 6: Real time Polymerase Chain Reaction page 12 of laboratory protocol booklet). **p < 0.01 ***p < 0.001 (vs. PBS) by Student’s t test (n = 6).

![FIG 9](image)

*Cell viability assessment by FACS. HepG2 cells were incubated in the absence (PBS) or presence of 20 μM native (HAS) or glycated albumin (AGE) for 24 hr. Cell viability was determined by FACS technique and following protocol no. 19 on page 40 of the laboratory protocol booklet. *p < 0.05 (vs. PBS) ###p < 0.01 (vs HSA) by Student’s t test (n = 7).

![FIG 10](image)

*Protein oxidation in liver or cell homogenates. Carbonylated proteins were revealed by Western blot following protocol no. 20 (page 42 of the laboratory protocol booklet).
References