MASTER IN BIOTECHNOLOGY OF
ENVIRONMENT AND HEALTH
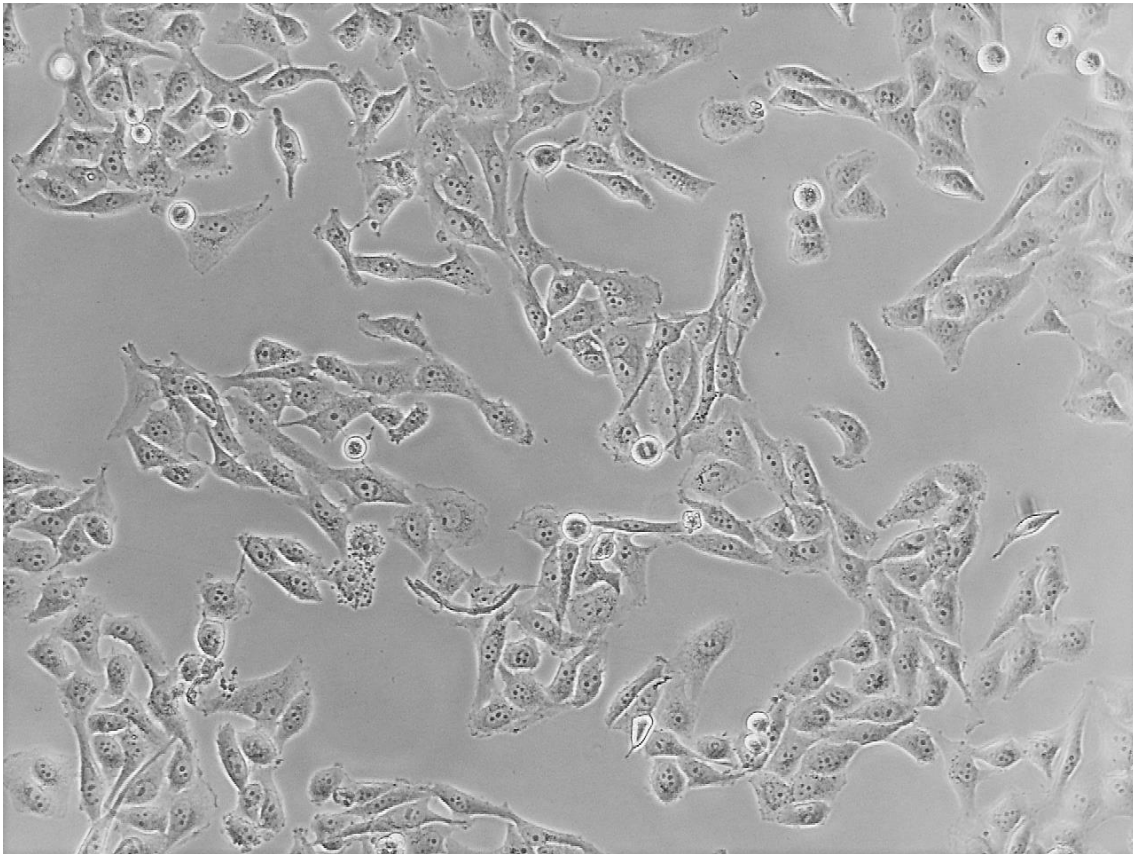
FINAL MASTER THESIS

THE TECHNOLOGY INFLUENCE ON THE STUDY OF OXIDATIVE STRESS

APPLIED TO BIOLOGICAL SAMPLES

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BIOQUOCHEM

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The research entitled "THE TECHNOLOGY INFLUENCE ON THE STUDY OF OXIDATIVE STRESS APPLIED TO BIOLOGICAL SAMPLE" has been made under my supervision by Ms. Susana Covadonga Rey Alonso graduated in Biology at Oviedo University in my laboratory of Bioquochem SL (Technology Park of Asturias-Llanera, Spain).

I hereby state that I have read and corrected the present Master Thesis document that I find suitable for its public defence by the student before the designed tribunal. Therefore, I authorize the submission of this Master Thesis to the University of Oviedo, MBEH academic commission.

In Llanera, 17 of July 2018

Signed: VºBº:
Dr. David Hevia Sánchez   Prof. Dr. Francisco Parra

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SUMMARY

Oxidative stress has been described as an imbalance between free radicals and antioxidants that is present in many diseases as cancer, among others. The increase on highly reactive species as oxygen derived species, cause damage to many biomolecules as DNA, lipids and proteins, with harmful consequences. This have raised the interest on the measurement of the oxidative status on many in vitro *as well as* in vivo *media. Many methods have been developed to measure free radicals as well as antioxidant capacity, which are inversely proportional. However, most of these classic methods present many differences and limitations, hindering comparisons among the methods. Specially, prostate cancer cells require this oxidative stress to proliferate and migrate, and even present the ability to produce hydrogen peroxide, a highly toxic free radical and resist its effects. On this work, a new electrochemical methodology is applied to determine its effectivity to measure differences on the redox responses of three cell prostate lines (PNT1A, LNCaP and PC3) to three treatments covering from hormonal to redox and metabolic alterations. The performance of this new method is contrasted with other classic techniques as FRAP and thiols measurement. To test possible applications of the method* in vivo*, a study with 43 volunteers was carried out to evaluate the antioxidant capacity of blood samples. From this work it can be concluded that the patent pending method and device eBQC is a promising tool for the evaluation of antioxidant capacity* in vitro *as well as* in vivo.

RESUMEN

El estrés oxidativo es el resultado de un desequilibrio entre radicales libres y antioxidantes que tiene lugar en un amplio abanico de enfermedades como puede ser el cáncer, entre otras. El incremento de las especies altamente reactivas como pueden ser las derivadas del oxígeno causa daños en las biomoléculas como el DNA, lípidos y proteínas con consecuencias peligrosas. Esto ha hecho aumentar el interés en la medida del estado oxidativo en varios medios tanto in vitro *como* in vivo. *Muchos métodos han sido desarrollados para medir los radicales libres y la capacidad antioxidante, dos parámetros inversamente proporcionales. Sin embargo, muchos de estos métodos clásicos presentan diferencias entre ellos y limitaciones, dificultando las comparaciones entre los mismos. En especial, las células de cáncer de próstata requieren este estrés oxidativo para proliferar y migrar e incluso son capaces de producir peróxido de hidrógeno, un radical libre altamente tóxico, y resistir sus efectos. En este trabajo, un nuevo método electroquímico ha sido aplicado para determinar su efectividad para medir diferencias en las respuestas redox de tres líneas celulares (PNT1A, LNCaP y PC3) a tres tratamientos que cubren desde alteraciones hormonales, redox y metabólicas. El rendimiento de este nuevo método se ha contrastado con otras técnicas clásicas como la medida de FRAP y tioles. Con el objetivo de probar posibles aplicaciones de este método* in vivo*, un estudio con 43 voluntarios se ha llevado a cabo para evaluar la capacidad antioxidante en muestras de sangre. De este trabajo se puede concluir que el método pendiente de patente y equipo eBQC es una prometedora herramienta para la evaluación de la capacidad antioxidante tanto* in vitro *como* in vivo.

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1. INTRODUCTION

Oxidative stress and its role in disease

Free radicals are highly reactive species that are naturally formed during cell metabolism in the mitochondria, secretion by immune cells during inflammation processes or taken up as environmental pollutants. Those substances can be either based on oxygen (ROS) for example O_2^-, HO, HO_2 and H_2O_2; or nitrogen (RNS) such as NO, ONOO, NO_2 and N_2O_3. These species are instable due to the presence of unpaired electrons which make them oxidize other compounds starting chain reduction-oxidation reactions that can lead to damage in vital biological substances as lipids, nucleic acids and proteins and so causing disease [1]. However, living organisms have developed systems to cope with the constantly produced free radicals, oxidizing themselves before other molecules and so protecting them: the antioxidants. While low concentration of free radicals presents a beneficial role as signaling systems, when produced at a higher rate than antioxidants, there is an imbalance referred to as oxidative stress [1].

In the last decades, chronic diseases have increased due to changes in modern lifestyle, especially regarding dietary habits. Alzheimer [2] and Parkinson disease [3], aging [4,5], cataract [6], and cancer [7,8], among others, are found to be correlated to oxidative stress events within the organism. Regular intake of foods with high antioxidant content such as fruits and vegetables have been shown to reduce the risk of suffering the previously mentioned diseases [9–12].

Lipids damage

When lipids are damaged by free radicals in cell membranes and lipoproteins, they form highly toxic byproducts as MDA and 4-HNE aldehydes in a process referred to as lipid peroxidation. Those molecules have been associated to processes on neurodegenerative diseases [13,14], diabetes [15,16], cardiovascular diseases [17,18] and cancer [19,20].

DNA damage

DNA can be oxidized by ROS being especially susceptible to OH$^\bullet$ one-electron oxidants and HOCl, resulting in mutagenesis and genetic instability (21). One of the most characteristic reactions leads to the formation of 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG that results in G:C to T:A changes. As a consequence of DNA damage, more ROS are generated, and the regulation of p53 activity is altered (22,23).

Proteins damage

Oxidation of proteins can be reversible or not and is important to regulate their activity or protect them from degradation. One of the irreversible processes under oxidative stress conditions is protein carbonylation, which produces aldehydes or ketones from side chain amino acids. The degree of carbonylation is considered to be an indicative of oxidative damage (24).
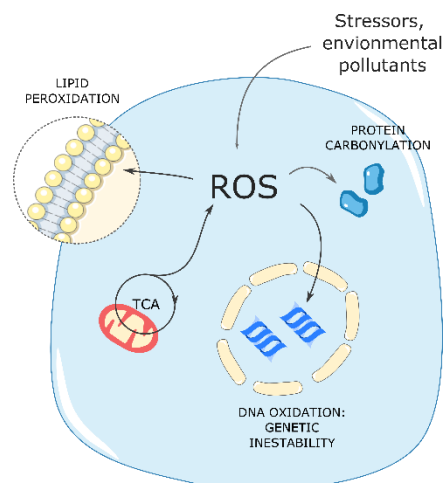
Figure 1. Scheme of the oxidative stress mechanisms.

Antioxidants and their classification

Antioxidants are compounds that neutralize free radicals by donating electrons but staying stable after these reactions, consequently preventing oxidative stress damage. Different classification criteria have been used according to structure, origin, kinetics or solubility. Structural classification, the most common, sort antioxidants as following:

- **Non-enzymatic antioxidants**: molecules that prevent oxidation of other compounds by donating their own electrons. Some examples are GSH, vitamin E, ascorbic acid, uric acid, polyphenols, bilirubin and albumin.

- **Enzymatic antioxidants**: enzymes that collaborate to catalyze the transformation of various types of ROS into hydrogen peroxide and finally into H_2O. Some of the more described enzymatic antioxidants are SOD (superoxide dismutase), CAT (catalase) and GSHpx (glutathione peroxidase).

According to their origin they can be classified as endogenous or exogenous, for example GSH endogenous production by the liver together with enzymatic antioxidants

constitute the main barrel against oxidative stress, while polyphenols and ascorbic acid constitute a great part of our daily antioxidant intake.

As mentioned above, free radicals are able to damage either water soluble compounds as well as lipids, so different hydrophilic and lipophilic antioxidants need to be generated, such as vitamin C and polyphenols (hydrophilic) or vitamin E and A (lypophilic) depending on their action site.

Respect to their kinetics, antioxidants can be considered either fast or slow, which may also be described as low molecular weight (LMWA) and high molecular weight antioxidants (HMWA). From those, the first ones present more biological importance given that they are easily oxidizable, having great protection potential.
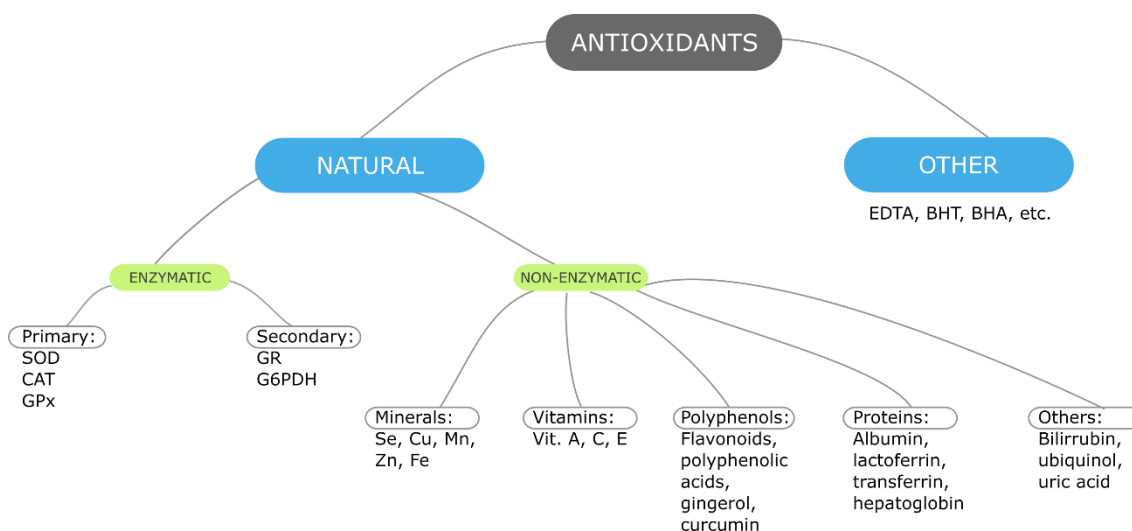
Figure 2. Scheme of the general classification of antioxidants. Modified from (25).

Oxidative stress mechanisms in prostate cancer

Alterations in the redox balance have the ability to modify transduction systems and even gene expression. Free radicals such as ROS and RNS play a key role on the disease. Even though there is scientific evidence of this alterations, a great part of its function remains unknown as well as its scope and importance.

Specifically, prostate cancer is one the tumors where more studies regarding oxidative state have been developed. It has been shown that there are variations in the expression and activity of enzymes with redox activity, in this way, recent scientific studies have proposed an association between the redox protein TRX1 and the Gleason

grade (26). Moreover, polymorphisms of the catalase enzyme have been described that increase the risk of prostate cancer (27) and even that certain polymorphisms of SOD and myeloperoxidases increase both the incidence of prostate cancer and its metastasis (28). On the other hand, it has been shown that a lower activity of SOD1, CAT and GPX in prostate cancer versus benign hyperplasia (29). PRX3 (Peroxiredoxin-3) levels increased in high degrees of Gleason vs benign hyperplasia tissues (30) have also been found and as this enzyme is overexpressed in prostate cancer conferring greater resistance of tumor cells to treatments (31).

Despite the lower activity of antioxidant enzymes, higher resistance to oxidative stress on prostate cancer cell lines like PC3 and LNCaP have been demonstrated (21) raising interest on the antioxidant mechanisms used. Hormonal, free radicals and accelerated metabolism factors are characteristic of prostate cancer as well as important on tumor oxidative stress balance.

Androgens play a key role on androgen dependent prostate cancer, promoting cell proliferation but has been demonstrated that they also increase oxidative stress status and radiation therapy resistance (32).

Hydrogen peroxide, one of the most relevant ROS, has been linked to opposite effects on cancer pathogenesis, on one hand, carcinogenic cell overproduction was observed due to the NADPH oxidases activity (33,34),but on the other, toxicity of the compound was correlated with apoptosis induction (35,36).

N-acetylcysteine (NAC) is a derivative of the amino acid cysteine and stands out by its antioxidant properties against ROS. Specially, NAC is able to counteract the effects of H_2O_2 not only in a direct manner (37) but also through other cellular signaling pathways (38–40), showing also antimetastatic potential (41).

Glucose metabolism is also altered during prostate cancer. GLUT transporters overexpression facilitates the uptake of not only glucose but also antioxidants as uric acid which helps the cells resist oxidative stress (42,43).

Measuring Antioxidants (TAC): Classical assays

Due to the different nature of antioxidants, evaluating the concentration of a single molecule or even of each antioxidant substance that constitutes a sample is not usually considered as representative of its ability to scavenge free radicals. Some molecules present a higher tendency to donate their electrons than others despite their lower concentration, enzymatic activity would be underestimated and finally, antioxidants may interact among them to reach higher efficiency. This is the reason that a common way to measure it is the Total Antioxidant Capacity (TAC), in which only the ability to neutralize free radicals is determined without taking into account each individual antioxidant component that constitutes the sample.

While more than 25 methodologies for the determination of TAC have been described, only a few of them will be explored in this work.

Antioxidant methodologies can be sorted according to the type of reaction that takes place:

- **Hydrogen Atom Transfer reactions (HAT)**: the hydrogen atom donating ability of the antioxidant is measured.
- **Electron Transfer reactions (ET)**: simple redox reaction takes place between the antioxidant and the free radical frequently leading to a change in the absorbance spectrum that is directly proportional to the TAC of the antioxidant sample.
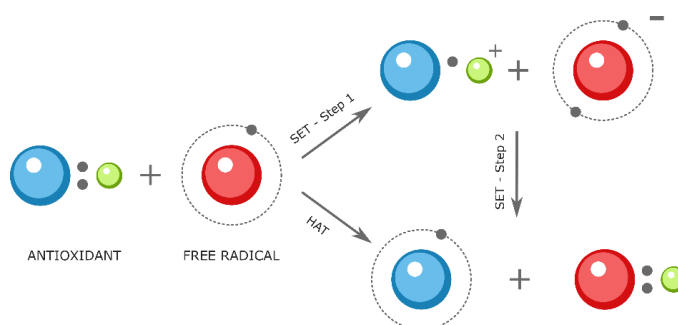

Figure 3. Scheme of the types of antioxidant reactions to scavenge free radicals. Modified from (44).

Hydrogen Atom Transfer Methods

ORAC

The ORAC assay depends on the free radical oxidation of a fluorescent probe, such as fluorescein, to result in a directly proportional decrease of the fluorescence. When an antioxidant is present in the sample, neutralizes the free radical before it damages the probe and so, preserving the fluorescence emission.

$$AAPH + ROO^{\bullet} + fluorescein \rightarrow non\text{-}fluorescent\ product$$
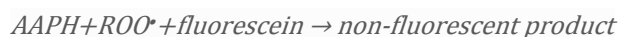
This assay is one of the most applied method to measure the antioxidant capacity mainly in food samples. ORAC uses peroxyl radicals as its free radical model which mimics best biological conditions with continuous radical generation. In addition, lipophilic and hydrophilic samples may be used with slight protocol modifications (45). It became a reference technique that even the USDA (United States Department of Agriculture) published a database containing ORAC values for thousands of food types.

However, on 2012, the organisation withdrawn the data from public access due to inconsistencies on the methodologies applied (frozen/dried/wet fruits were evaluated at the same level) (46) and biological relevance of food ORAC values and difficulties on the results interpretation were questioned (47).

Electron Transfer Methods

DPPH

DPPH assay for the use of antioxidant capacity determination covers pure substances, food samples, solids and liquids. In methanol solution it gives rise to a purple colour that disappears when in contact with an antioxidant substance.

$$DPPH\ (purple) + AOH \rightarrow DPPH_2 + AO\ (yellow)\ (\lambda max = 515\ nm)$$
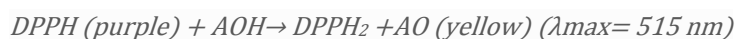
This assay is one of the most common to be routinely done in laboratories, being considered one of the most stable free radicals. Though, the assay has overcame different adaptations over the years with no standardized methodology making comparisons difficult (48). Technically, environmental factors have a great effect on DPPH reactions mainly pH, O_2, light exposure and the solvent used. For example, methanol, the most common used solvent, inhibits the DPPH scavenging while aqueous solvent favour it (45,48).

FRAP Antioxidant Capacity Assay

Ferric Reducing Ability of Plasma assay measures the total antioxidant capacity of various biological samples and beverages. It is based on the reduction of the complex TPTZ-Fe^{3+} at acidic pH in the presence of an antioxidant, developing a purple colour directly proportional to the concentration of the antioxidant that is measured at 593 nm. Usually, the antioxidant capacity is measured as equivalents of Fe (II) mM.

$$Fe^{3+}\text{-}C + AOH \rightarrow Fe^{2+}\text{-}C \text{ (blue)} \text{ } (\lambda max = 593 \text{ } nm)$$
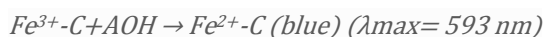
FRAP is a fast, simple and inexpensive method to measure antioxidant capacity with great reproducibility and no specialized equipment (49). This assay has the limitation that is not able to measure enzymatic and thiol groups antioxidant activity. Another consideration is that the determination is taken under non-physiological conditions may not reflect the substance biological behaviour (50).

TEAC

TEAC (Trolox Equivalent Antioxidant Capacity) assay is used for the measurement of total antioxidant capacity of pure substances, aqueous solutions and beverages. The working fundamental of this assay is the neutralization of a coloured free radical, in this case, the synthetic $ABTS^{\cdot+}$ (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). The standard used is Trolox, a compound with a similar structure to vitamin E.

$$ABTS \text{ (uncolored)} + Reagent \rightarrow ABTS^{\cdot+}(blue/green) \text{ } (\lambda max = 734 \text{ } nm)$$
$$ABTS^{\cdot+}(blue/green) + AOH \rightarrow ABTS \text{ (uncolored)} + AO$$

One of the main advantages of this method is its flexibility given that it shows good solubility on both aqueous and organic media. In addition, the assay is fast, simple and works within a wide pH range. However, it has been discussed about how the free synthetic radical is able to mimic natural ROS/RNS activity on the body due to its large structure (45) and how the products of the parental antioxidant substance show the ability to overestimate TEAC measurement (51). Furthermore, there exists different methods of generating the free radical which may also influence the results (50).

Limitations of TAC classic methodologies

TAC tests conditions vary considerably among them (pH, medium, temperature) as well as the structure of the molecule to be reduced by the sample (size and complexity),

the standard and therefore the final units of TAC measurement (Trolox Equivalents, Ascorbic Acid Equivalents, Gallic Acid Equivalents and others). This makes that the same sample give rise to different antioxidant capacity results on different tests. Even with the same units, the methodologies are still not comparable, which makes that antioxidant capacity should be obtained from different techniques.

The main limitation of the classical TAC methods is that they tend to measure only the non-enzymatic component, ignoring the potential of enzymes, and in many cases thiols (and therefore glutathione).

While HAT and SET based methods differ on the chemical reactions, they also show different performance according to the solvent, pH and reaction time used. Alcohol based solvents and higher pH tend to favour SET reactions while water-based solvents impulse HAT. Also, low reaction times lead to the measurement of ET processes while HAT processes tend to take longer times to inhibit free radicals damage, usually underestimating the antioxidant capacity of the sample. Moreover, most of these assays present greater application on *in vitro* studies rather than *in vivo* ones, which may decrease its biological significance.

Measuring oxidative stress: Current assays

Apart from the TAC determination methodologies, there exist other techniques to measure oxidative stress by determining the number of damaged biomolecules on the sample. A brief description is given below:

LPO

Lipid peroxidation is measured by determination of the concentration of MDA and HNE on the sample. Reactions between indoles with the MDA and HNE results in a product that can be measured at 580-620 nm.

$$Reagent\ A + MDA \rightarrow C\ (\lambda máx = 586\ nm)\ Reagent\ A + HNE \rightarrow C\ (\lambda max = 586\ nm)$$
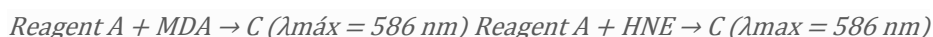
MDA/TBARS

The Thiobarbituric Acid Reactive Substances (TBARS) assay is a tool for the direct quantitative measurement of MDA in biological samples. It is based on the reaction of TBA with MDA that can be read spectrophotometrically or fluorometrically. TBARs assay is commonly used to compare one set of samples to another.

$$\text{2-Thiobarbituric Acid + MDA} \rightarrow \text{MDA-TBA Adduct } (\lambda max = 532 \text{ nm})$$

Thiols

This assay measures the reduced and oxidized thiol groups in biological samples using Ellman´s Reagent, which reacts with GSH, generating a colorimetric signal at 412 nm

$$\text{R-SH + DTNB} \rightarrow \text{R-TNB + TNB (Yellow) } (\lambda max = 412 \text{ nm})$$

$$\text{R-S\_S-R' + NaBH4} \rightarrow 2 \text{ R-SH + BH3+ Na}$$

Protein carbonylation

This assay is designed for the measurement of oxidized proteins as an index of oxidative stress. It is based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl groups giving a product that presents a peak of absorbance at 365–375 nm.

$$\text{2,4-dinitrophenylhydrazine (DNPH)+ Carbonyl} \rightarrow \text{Imine (hydrazone) } (\lambda max = 365\text{-}375 \text{ nm})$$

Enzymatic activity assays

The activity of various enzymatic antioxidants can also be tested through different assays such as SOD, CAT, and GSHpx to evaluate their ability to remove their substrate from the sample. Low enzymatic levels indicate less enzymatic expression leading to oxidative stress conditions.
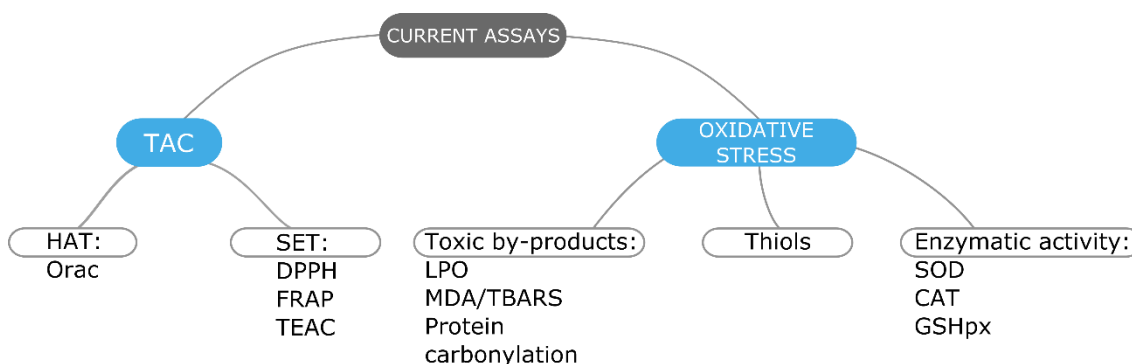
Figure 4. Scheme of the current methods of the determination of oxidative stress.

eBQC: Development of a new electrochemical method

As have been proved above, there exists the necessity for new methodologies that can overcome the drawbacks of the current assays and stablish a standardized technique able to precisely, fundamentally correct and biologically relevant, determine the antioxidant capacity *in vitro* as well as *in vivo*.

While electrochemical methods have already been described, they are not widely implemented. From Bioquochem, an electrochemical device to measure TAC has been designed. This method presents the advantage that it is able to distinguish from fast and slow antioxidants, and contrary to the previously mentioned technologies, it is capable of measuring thiols.

2.   OBJECTIVE:

Oxidative stress plays a key role on different pathologies, especially on the balance of cellular homeostasis. Some of those diseases, as cancer, an imbalance between ROS generation and antioxidant is produced. However, this is difficult to be measured and new technologies need to be developed to quantify oxidative levels *in situ*. Given that some authors have questioned the methodological part used for the evaluation of the redox status, the aim of this work will be to develop a new method to evaluate oxidative balance in a precise, fast and sensitive manner in prostate cancer cells and in human samples. The main objective was divided on the following specific ones:

1. To study the effects of redox alterations on tumoral and non-tumoral prostate cells.
2. To evaluate the different redox parameters levels on tumoral and non-tumoral prostate cells with classic technology.
3. To develop and test a new redox measurement technology on tumoral cells *in vitro* and in human samples *in vivo*.

3.   MATERIALS AND METHODS

Materials

Hydrogen peroxide at 30% purity (Ref. *216763*), HEPES (Ref. *H3375*), Trypsin (Ref. *T4549*) and Fetal Bovine Serum (FBS) (Ref. *F7524*) were purchased from Sigma Aldrich. Rosewell Park Memorial Institute 1640 (RPMI-1640) (Ref. *BE12-167F*) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12) (Ref. *BE12-707F*) culture mediums were purchased from Lonza. Dihydrotestosterone (DHT) or 5-α-androstan-17-β-OL-3-ONE was purchased from Fluka (Ref. *10300*). N-Acetyl-cysteine (NAC) (Ref. *A15409*) was purchased from Alfa Aesar. L-glutamine 200 mM (Ref. *SH30034.02*) was purchased from HyClone. 99 % Ethanol (Ref. *0321F*) was purchased from Alcoholes Montplet. The antibiotic/antimycotic used was a mixture of penicillin, streptomycin and amphotericin B (Ref. *L0010-100*) was purchased from BioWest.

MTT Cell Proliferation Assay Kit (Ref. *KF03001*), DNA Quantitation Kit – Hoechst Assay (Ref. *KC04003*), DCFH-DA Probe (Intracellular ROS assay) (Ref. *KP06003*), FRAP Antioxidant Capacity Assay Kit (Ref. *KF01003*), Thiol Quantification Assay Kit (Ref. *KB03007*), Lipid Peroxidation Assay Kit (Ref. *KB03002*), and Bradford Protein Assay Kit (Ref. *KB03003*) were from the brand BQCkit.

Cell cultures

Three cell lines were used in the experiments: two prostate cancer cell lines LNCaP and PC3, and PNT1A as a control.

LNCaP are androgen-dependent human prostate cancer cells that were isolated for the first time in 1977 from a 50-year-old Caucasian male from the metastatic left supraclavicular lymph node by needle aspiration biopsy (52). Those are epithelial adherent cells presenting slow growth and a tendency to form clusters. The cells were defrosted and cultured in a 75 cm^3 flask with 20 ml of RPMI 1640 medium containing 10 % FBS, 0.3 g/L glutamine and 1% of an antimycotic-antibiotic cocktail.

PC3 are androgen-independent human prostate cancer cells that were initiated from a bone metastasis of a 62-year-old Caucasian male in 1979 (53). This cell line is epithelial and adherent and forms clusters. The cells were defrosted and grown in DMEM-F12

medium containing 10 % FBS, 0.3 g/L glutamine and 1% of an antimycotic-antibiotic cocktail.

PNT1A is a normal prostatic epithelial cell line that was isolated from a 35-year-old man at post mortem and immortalized through transfection with a plasmid containing SV40 genome, which has a defective replication origin (54). The cells were cultured in a 75 cm^3 flask with RPMI 1640 medium containing 10 % FBS, 0.3 g/L glutamine and 1% of a antimycotic-antibiotic cocktail.
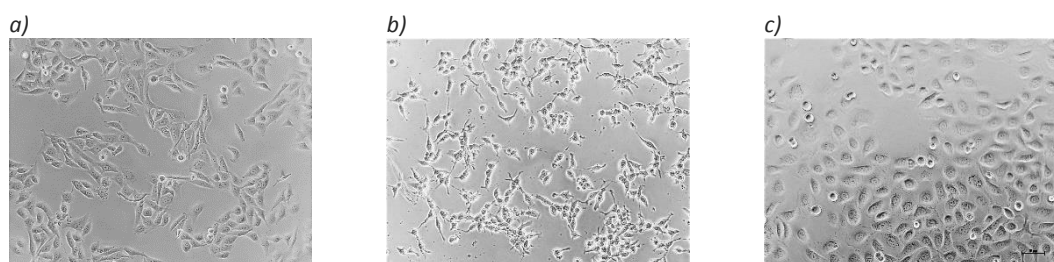

*Figure 5. Micrographs (10x) of the celular lines used on this work **a)** PNT1A; **b)** LNCaP; **c)** PC3*

Cell seeding

Cytotoxicity assays were performed in three different 96 wells plate for the three methods (MTT cellular activity assay, Hoechst cellular proliferation assay and DCFH-DA ROS activity assay). Cells were seeded at a density of 25000 cells/ml in the case of PNT1A and PC3 and 50000 cells/ml in the case of LNCaP due to its slower growth rate. To obtain the protein samples, cells were seeded in 100 mm culture dishes in the case of PNT1A and LNCaP and in 6-well plates in the case of the PC3 cell line.

Treatments applied

Three treatments were tested against a control in both the 96-well plates and the culture dishes for the three cell lines. DHT was added to a final concentration of 10 nM. In the second treatment 1 mM of NAC and 150 µM of H_2O_2 was added, and finally, glucose was added to the medium to double its normal concentration.

Same concentrations were used for the culture dishes with the exception of NAC and H_2O_2 that were added to a concentration of 100 mM and 300 µM respectively.

Cell harvesting and culture medium collection

One ml of culture medium of PNT1A and LNCaP cells was collected and stored at

-20°C until the day of the assay, while 0.5 ml were collected in the case of the PC3 line. The cells in the plates were harvested through scraping with the rest of the culture medium. The cells were centrifuged for 5 minutes at 500 g and 4°C. The supernatant was removed. A washing step with 1 ml of cold PBS was done and the sample was centrifuged another 5 minutes at 500 g and 4°C. The PBS was removed to store the cells in dry pellet at -20°C.

Protein extraction

Protein extraction buffer was freshly prepared consisting on RIPA buffer containing 1 mM of PMSF, 0,02 mM DTT and 2 µg/ml apoprotein A, 10 µg/ml leupeptin, 1 mM NAF, sodium orthovanadate 200 µM and 1 µg/ml pepstatin as protease inhibitors.

Seventy-five µl of the protein extraction buffer was added to PNT1A and LNCaP while for PC3 15 µl were added and let incubate for 30 minutes at 4°C. After the incubation period, samples were centrifuged at 15000 g at 4°C for 15 minutes. Supernatant was collected and stored at -20°C.

Protein quantification

Quantification of the amount of protein extracted was carried out with the Bradford assay using diluted BSA in the protein extraction buffer as a standard. Two µl of the extracted protein or the BSA standard solution, 500 µl of Milli-Q H_2O and 500 µl of Bradford Solution were mixed in an microtube. Two hundred µl of the solution were transferred to a 96- well plate by duplicate and measured the absorbance at 595 nm in a microplate reader.

MTT assay

Forty-eight hours after the addition of the treatment, 10 µl of the MTT solution was added to each well and incubated for 4 hours at 37°C in a culture hood. After incubation, the culture medium was removed and 100 µl of the MTT Solvent were added to each well which corresponds to the original culture volume. The plate was covered and agitated for 15 minutes before reading the absorbance at 570 nm.

Hoechst assay

The reagents were prepared according to kit instructions. After 48 hours since treatment addition, the medium was removed by overturning the plate on absorbent paper towel and the plate was frozen at -80°C for at least one day. After this period, 100 µl of Reagent A were added to each well and the plate was incubated during 1 hour at 37°C followed by another day of storage at -80°C. The plate was next thaw it until reaching room temperature and 100 µl of DNA marker solution were added to each well. The plate was next covered and agitated in an orbital shaker before measuring fluorescence ($\lambda_{excitation}$: 350 nm/ $\lambda_{emmision}$: 460 nm).

DCFH-DA assay

The protocol followed in this case was for a microplate reader and adherent cells. The reagents were prepared according with the kit instructions immediately before use. Solution reagent was diluted to a final concentration of 20 µM. Culture medium was removed and cells were washed with 100 µl of Reagent A before the addition of 100 µl of the Probe Working Solution. The plate was incubated for 60 minutes in dark conditions at 37 °C. After the incubation period the medium was removed and 100 µl of PBS were added right before measuring fluorescence ($\lambda_{excitation}$: 485/ $\lambda_{emmision}$: 535 nm).

FRAP assay

Culture medium antioxidant capacity was evaluated. The FRAP Working Solution was prepared immediately before its use. 10 µl of sample or Fe (II) standard were added to each well and 220 µl of the freshly prepared Working Solution and mixed under continuous stirring for 4 minutes just before measuring its absorbance at 593 nm.

Thiols assay

Thiols assay was done with both culture medium and protein extracts that were tested in duplicates. For the protein extracts only 1 µg of protein was used for each sample. The reagents were freshly prepared as indicated on the kit instructions. The procedure used in this kit was to measure the native free thiols. Twenty µl of sample or the standard were added to each well together with 20 µl of the Reagent A and the

reaction was left for 10 minutes. Two hundred and twenty µl of Reagent C were added and left another 10 minutes to read absorbance at 412 nm.

LPO assay

The lipid peroxidation assay was carried out on cell extract samples containing 1 µg of protein. The reagents were prepared as indicated on the kit instructions. Every sample was run in duplicates. 100 µl of sample (containing 1 µg of protein) were mixed with 325 µl of Reagent A, 75 µl of Reagent B and incubated for 40 minutes at 40°C. Two hundred µl of each microtube were transferred to a 96 well-plate and the absorbance was measured in a microplate reader at 586 nm.

eBQC assay

Antioxidant capacity measurements with the eBQC device (Figure 6) were done with culture medium, protein extracts and freshly obtained human blood samples. In the first case, 30 µl of every culture medium sample were used. Due to the low volumes of sample for the protein extracts it was necessary to create a pool of proteins for each combination of cell and treatment with a final concentration of 0.1 µg of protein/µl diluted in Milli Q H_2O. In this case, the assay was done in triplicate using 30 µl of the diluted protein. Also, human blood samples were collected from 43 volunteers. Results for charge of the slow and fast antioxidants as well as the total charge of antioxidants were noted.

Figure 6. eBQC device and disposable strips

Data analysis

The data analysis was done with R Studio (55) and the packages car (56), moments (57) and ggplot2 (58). Before proceeding to analyse the response differences among groups, normal distribution and skewness of the residuals was tested through the Shapiro-Wilk normality test and homoscedasticity was tested through Fligner-Killen test and was evaluated.

For normally distributed data, one-way ANOVAs were performed for each assay and cell line. In the case significant differences were found, *post hoc* analysis was run, being Tuckey HSD the one used on this work. For all the analyses, a P value of <0.05 was used to determine statistical significance.

4.   RESULTS

Cytotoxicity/Viability of the treatment: MTT and Hoechst assay

The control cellular activity measured by MTT was expressed as a percentage, considering the control group a 100 %. While no significant differences were observed on the MTT cellular activity among treatments for PNT1A cell line, it can be observed a decrease on LNCaP and PNT1A cells under the H_2O_2 with NAC treatment (see Figure 7a).
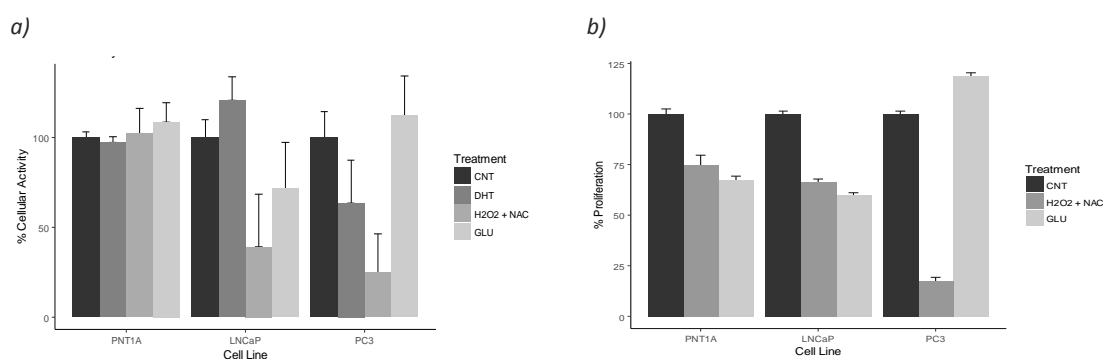

Figure 7. **a)** Cellular activity adjusted to control levels measured by MTT, **b)** Cell proliferation adjusted to control levels measured by Hoechst.

The results of the Hoechst assay (refer to Figure 7b) show similar results to those obtained for the MTT assay, especially on the PC3 cell line. In the case of PNT1A and LNCaP similar results were obtained for the H_2O_2 with NAC and glucose treatments, with significant differences to the control.

Generation of ROS by the cells under each treatment: DCFH-DA assay

ROS generation was lower on the H_2O_2 + NAC group respect to the control on every cell line (p-value= 0.007, $1\cdot10^{-4}$ and $1\cdot10^{-6}$). PTN1A and PC3 had a similar response on the glucose treatment with lower ROS activity (p-value= 0.001 and 0.0001 respectively) while LNCaP showed no differences with the control (see Figure 8). The results of ROS activity were correlated with the cell survival as can be seen on the Figure 8c, 2d and 2e as an example for PC3 response.

a)
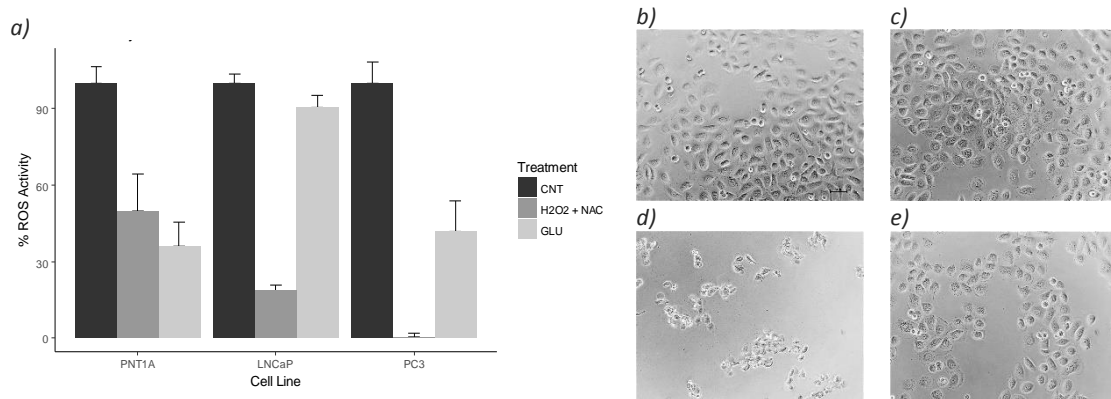
*Figure 8. **a)** Relative ROS Activity adjusted to control levels measured by DCFH-DA fluorescent probe; Micrographs (10x) **b)** PC3 cells before treatment; **c)** PC3 control group after 24 h; **d)** PC3 H_2O_2 + NAC group after 24 h of treatment; **e)** PC3 glucose control group after 24 h of treatment.*

Culture medium antioxidant capacity: FRAP assay

On the FRAP assay for culture medium, antioxidant capacity was only observed on the H_2O_2 + NAC group. PC3 showed the highest levels and LNCaP the lowest (see Figure 9). Statistical differences were noted only for PC3 and LNCaP group with a p-value of 0.034.
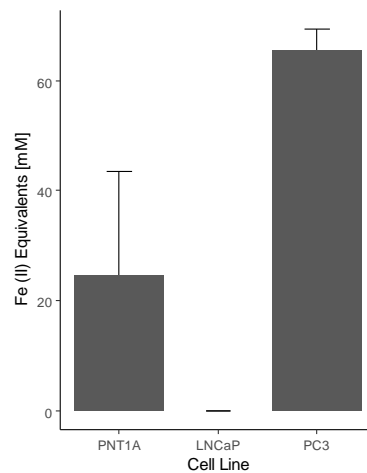
Figure 9. Results of the antioxidant capacity test FRAP for the H_2O_2 + NAC group

Thiol content on culture medium and protein extracts.

On the Figure 10a, no statistical differences were observed for any of the groups. The H_2O_2 + NAC group is plotted apart because the structure of NAC contains SH groups and interferes with the assay. On the Figure 10b, PC3 and PNT1A showed high levels of free thiols, being higher on PC3, while in LNCaP, the levels were almost undetectable.

On the Figure 10c only significant differences were observed for PC3 cells (p-value=$2.5 \cdot 10^{-5}$), on protein extracts, on which the free thiols increased with the H_2O_2 + NAC treatment, which is correlated with the free thiols on culture medium.

a)   b)

c)

Figure 10. Results of the free thiols measured on **a)** cell culture medium; **b)** culture medium for the H_2O_2 + NAC group; **c)** protein extracts.

Lipid peroxidation on protein extracts: LPO assay

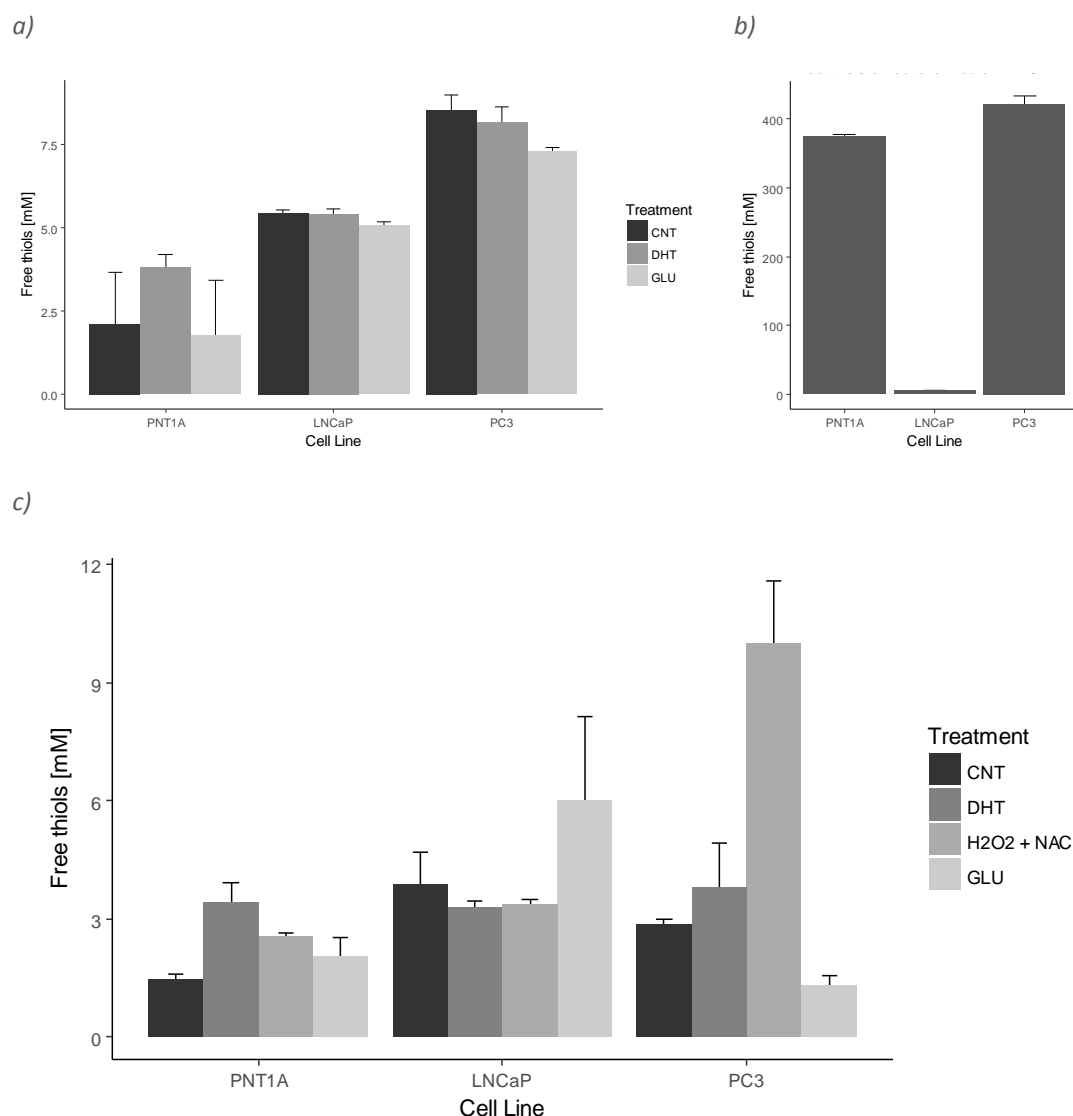Lipid peroxidation levels were below the detection levels of the assay (data not shown). This was due to the type of extraction and the low concentration of protein used for this assay.

Antioxidant capacity measured on culture medium and protein extracts: eBQC

Culture medium

On the eBQC assay, only statistical differences were observed for the fast antioxidants on PNT1A cell line regarding H_2O_2 + NAC group versus the control (refer to Figure 11).

Protein extracts

PNT1A have increased fast antioxidants under DHT treatment and a decrease in the H_2O_2 + NAC group which is considered statistically different and is maintained for slow and total antioxidants. No other differences were found among treatments on the same cell line regarding fast antioxidants. Although not significative differences, LNCaP have a little increase on H_2O_2 + NAC group fast and slow antioxidants. PC3 show an increase on slow antioxidants in the H_2O_2 + NAC group versus the control. In all glucose groups the antioxidant capacity is similar to the control (see Figure 11).

Total antioxidants are the sum of the fast and slow antioxidants, however slow antioxidants have a greater impact on the total antioxidants because they tend to be on higher concentration than the fasters.
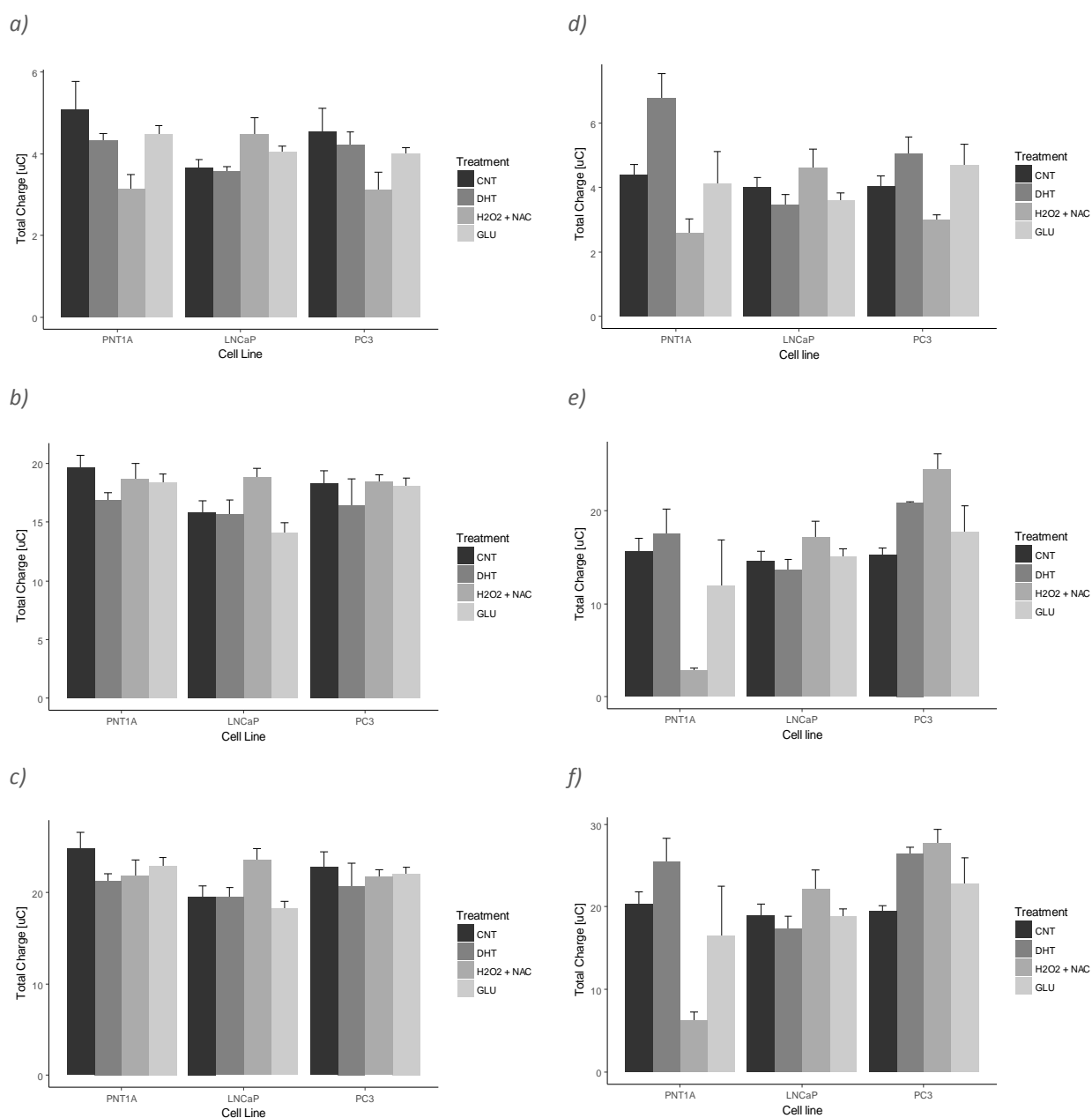
*Figure 11. Antioxidant capacity of culture medium sample: **a)** Fast fraction antioxidants; **b)** slow fraction antioxidants; **c)** total antioxidants; Antioxidant capacity of protein extracts: : **d)** Fast fraction antioxidants; **e)** slow fraction antioxidants; **f)** total antioxidants.*

In vivo assay

From the 43 volunteers, 10 were male and 33 women. The Table 1 summarizes the statistical data from the population. The distributions were slightly skewed to the right, except for the total antioxidants (0.038, 0.396 and -0,006 respectively for fast, slow and total antioxidants). No statistical differences were found on the antioxidants according to gender (p value=0.647) (see Figure 12d).

Table 1. Statistical data from the in vivo *study*

Antioxidant	Mean	Median	Min	Max
Fast	9.791	9.195	3.790	15.500
Slow	14.98	15.19	1.40	31.60
Total	25.19	25.80	15.00	38.29

a)

b)

c)

d)

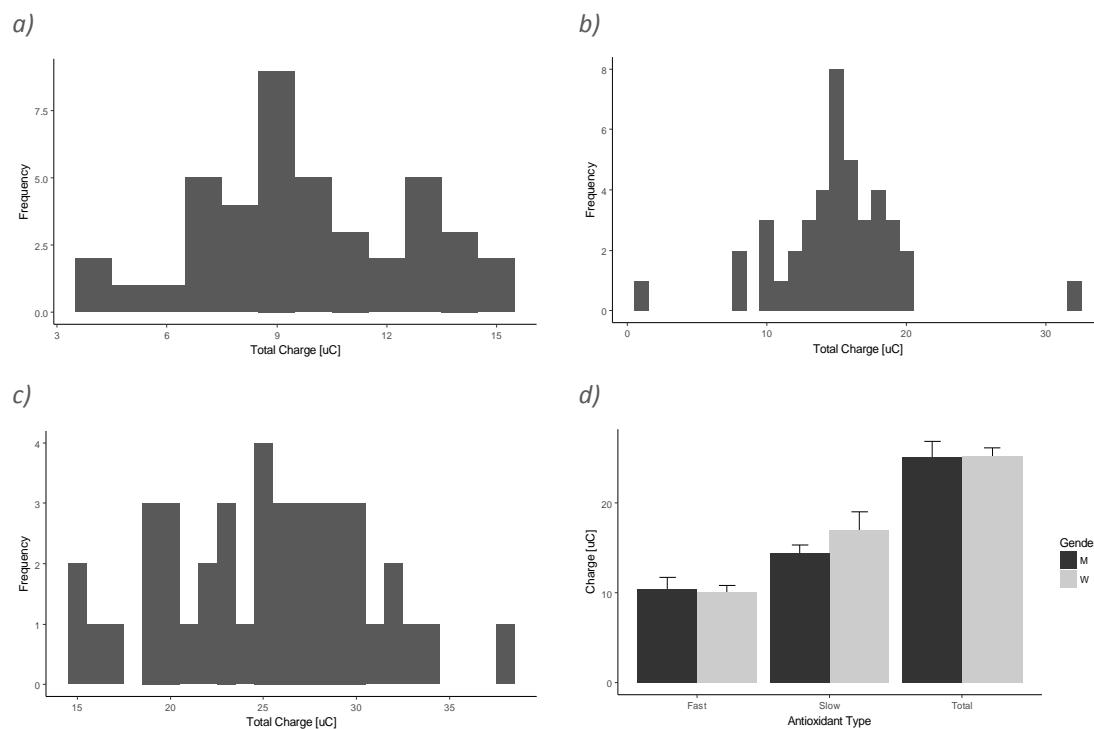*Figure 12. Population distribution of **a)** fast antioxidants; **b)** slow antioxidants; **c)** total antioxidants; and **d)** the differences observed between men (**M**) and women (**W**).*

5. DISCUSSION

Cytotoxicity: MTT y Hoechst

On the assays performed to change redox balance, mitochondrial activity and cell proliferation were evaluated. LNCaP cells responded to the androgens treatment by increasing their cellular activity though not significative, which could be expected since LNCaP cells are androgen-dependent (52). Contrary to what it might seem due to the androgen-independence of PC3 cells (53), they show a non-significant decrease on their growth, which have been described before on the literature as the result of an increase in the expression of neutral endopeptidase (NEP) (59). In the case of PNT1A, there are no observed differences.

Prostate cancer cells produce H_2O_2, which is even considered the fuel for cancer proliferation (60), and its production is directly proportional to the aggressiveness of the cells, being almost double on PC3 than LNCaP (21). However, there is a point where the hydrogen peroxide changes from increasing cell proliferation and migration to inducing cell death (35). Since PC3 tend to produce higher levels of hydrogen peroxide, they might be more affected on their survival than LNCaP and PNT1A by the addition of H_2O_2, which supports the results obtained on the Figure 7a and b. In combination with this factor, NAC has been described as an inhibitor of cancer cells growth and invasion properties (41).

While on many cases, MTT assay and Hoechst are considered to be equivalent, this is not completely true given that the MTT assay measures the mitochondrial activity and Hoechst the amount of DNA. The Hoechst assay presents some differences to the first one.

Oxidative stress - ROS generation: DCFH-DA assay

The combination of H_2O_2 and NAC caused a decrease of the free radicals, however, this is not representative of what was happening with the cells, which was the effect of high toxicity that led to cellular apoptosis (refer to Figure 7). The doses of H_2O_2 and NAC were too high and the exposure times too long. This decreases cell survival and so, oxidative stress is not able to be distinguished.

Increased ROS levels were expected for the glucose treatment, due to the increased mitochondrial activity, however unexpected lower results were obtained for PNT1A and PC3 cell lines. A possible explanation to this fact might be that at high doses of glucose, the cells tend to derivate a part of this glucose to the pentose phosphate pathway, that produces NADPH, which is a reducing agent itself, but also is a cofactor needed to catalyze the conversion of GSSG to GSH one of the most important antioxidants (61).

Antioxidant capacity: FRAP, Thiol assay and eBQC method

The antioxidant capacity assay FRAP presented high detection levels for this sample, being unable to detect antioxidant effects on any sample that had not NAC added. LNCaP cells show lower antioxidant capacity on this same group indicating that it might had been consumed. Those results agree with the free thiols measured on the NAC group, where LNCaP had almost not free thiols (Figure 10b). However, as mentioned above, FRAP methodology is considered to be uncapable of measuring antioxidants based on thiol groups (50) due to the slow reactivity of SH groups towards the ferric complex. This generalization is not completely true since as it has been demonstrated in this work and in some publications (62,63). Due to the differences on each compound kinetics, FRAP is able to measure NAC (Figure 9) and other thiol based antioxidants as DTE (dithioerythritol) and reduced lipoic acid (DHLA), whereas it cannot measure GSH, cysteine or homocysteine.

The interference from NAC on the assay together with the inability of FRAP to measure GSH which is the main antioxidant compound produced by the cells, makes it not appropriate to detect the antioxidant capacity of culture medium under these conditions.

On the other hand, the electrochemical method eBQC is able to discriminate NAC due to the parameters established on the voltammetry (since it starts the measurement at 0,3 V and NAC presents a lower oxidation potential) and it measures GHS as well. For that reason, contradictory results were found for the culture medium in comparison to those of FRAP. In spite of this, similar values were obtained for thiols and the eBQC on culture medium, presenting only differences on the H_2O_2 and NAC group. This demonstrates that on PNT1A and PC3 cells, the antioxidant capacity arises from the NAC

interference (refer to Figure 10 and Figure 11. LNCaP show thiol levels similar to those of the control, which may be derived from GSH or cysteine and its derivatives that are detectable through the eBQC method (slow antioxidants) but not through FRAP.

This correspondences between the eBQC and thiols measurements are also present on the protein extracts evaluated. An exception will be the PNT1A cells for the H_2O_2 and NAC treatment. These differences were caused by a higher density on the protein pool that reduced the conductivity of the sample causing the measurement to be lower than what it should be. In spite of this, good correlation was found between PC3 response between the thiols assay and eBQC, which might suggest that the cells were up taking part of the NAC on the medium, and that this antioxidant played a role on the protection of GSH and other reduced cysteines, which is also reflected on the slow antioxidants.

It has been demonstrated that PC3 generate high amounts of GSH and reduction of the Gl- Red (glutathione reductase) to resist the H_2O_2 that are producing (64). Since this has been proved for other cancer cell types and based on the results obtained, LNCaP may respond in a similar way to PC3 producing high amounts of GSH. However, LNCaP seems to behave differently than PC3 regarding antioxidant uptake, showing a faster consumption of NAC.

eBQC Patent and method

Given that the eBQC has been proved *in vitro* as well as *in vivo* on different sample materials and its efficacy has been proved, a patent with the device and method described on this work as the object of invention has been requested (number of request: P201830492).

As have been proven here, antioxidant capacity assays display contradictory responses due to the different parts of the TAC detected and the interferences from the NAC added and in many cases, the detection levels were above the presents on the sample. In addition, low reproducibility for the FRAP and thiols prevents the detection of significative differences. A new promising voltammetric method is able to measure thiols and a wide range of antioxidants at physiological pH, leading to a general and biological relevant determination of TAC on *in vitro* measurements as well as *in vivo*.

Moreover, it is able to distinguish between slow and fast antioxidants, an interesting feature not present on other TAC assays.

6.   FUTURE OUTLOOK

In view of the results and conclusions of this final master thesis, the study of redox balance will continue as indicated below.

As an initial point, some of the experiments will be repeated to confirm the results obtained on this work.

More assays of evaluation of TAC will be tested as DCFH-DA measured by flow cytometry, among others.

Lastly, broadening of the type of samples on which the eBQC could be applied as it could be urine, sweat or saliva will be tested. On this point, some preliminary results have been obtained with a small sample size.

Finally, this work will be continued since it is included on the Ministery Program of Talent Promotion and Empleability - Industrial Doctorates (Ref. BOE-A-2014-13664), to which the received funding is behold.

7.   CONCLUSIONS

From the results obtained on this work, the following conclusions can be drawn:

1. Hormonal, metabolic and antioxidant environment affect mostly to tumoral cells rather than normal prostatic cells.

2. Oxidative stress variations are quantified differently depending on the used methodology.

3. The device and method presented is viable to be used on cellular samples *in vitro* as well as human samples *in vivo*, allowing a generic measurement of oxidative status to be obtained.

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9.   ANNEX

The present work has been done on the company BIOQUOCHEM S.L. with its principal place of business located at Edificio CEEI. Parque Tecnológico de Asturias, under a confidentiality agreement. For this reason, there are parts that cannot be disclosed.