UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Master in Biochemistry

PROTEIN OXIDATION AND CIRCADIAN RHYTHMICITY: TOWARDS THE IDENTIFICATION OF SPECIFIC PROTEIN TARGETS

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ABSTRACT

The circadian rhythmicity exists in all organisms and generates rhythms with a periodicity of 24 h of a significant number of biological and metabolic processes. A disruption of the circadian clock (BMAL1 and PER2) in mice, are directly associated to an increase of reactive oxygen species (ROS) which induce a premature aging phenotype (Kondratov, 2007; Ramsey, *et al*., 2007). On the other hand, it was described that the accumulation of oxidized (carbonylated) proteins is a hallmark of cellular aging. Moreover, protein carbonylation, an irreversible modification, involves a decrease quality of the cellular proteome which could directly affect the normal cell function.

Using HEK-293 cells synchronized with serum shock and the 1D electrophoresis technique, our working group showed that the level of carbonylated proteins has a circadian rhythmicity. This quantification of the total proteins allowed to choose two peaks of the circadian rhythm corresponding to maximum and minimum levels of protein carbonylation. In this work 2D electrophoresis were also used, to characterise some particular proteins, which seem to suffer a circadian oxidation.

After quantification of carbonylated proteins, it was observed that the same spots are common all the times, even if their level of carbonylation is noticeably different. Concluding, it was proved, although without final characterization, that some specific proteins have a circadian oxidation. For the future, it will be interesting to identify these specific proteins and compare them to biological markers of oxidative stress in order to better understand the involvement of the circadian rhythm.

Keywords: Circadian Cycle; Serum shock; Dexamethasone; Carbonylated Proteins; Aging

RESUMO

O ritmo circadiano existe em todos os organismos e gera ritmos com uma periodicidade de 24 h em um significante número de processos biológicos e metabólicos. Uma disrupção do relógio circadiano (BMAL1 e PER) em ratos, está diretamente associada com o aumento das espécies reativas de oxigénio (ERO) que induzem um fenótipo de envelhecimento prematuro (Kondratov, 2007; Ramsey, *et al*., 2007). Por outro lado, foi descrito que a acumulação de proteínas oxidadas (carboniladas) é um marcador do envelhecimento celular. Para além disso, as proteinas carboniladas, com modificação irreversível, envolve uma diminuição da qualidade do proteoma celular que pode afectar diretamente a função da célula normal.

Através da utilização de células HEK293 sincronizadas com *serum shock* e utilização das técnicas de electroforese de ima dimensão, o nosso grupo de trabalho demonstrou que o nível das proteínas carboniladas tinham uma ritmicidade circadiana. Essa quantificação do total de proteínas levou-nos a escolher dois picos do ritmo circadiano, correspondentes ao máximo e mínimo de nível de carbonilação. Neste trabalho, utilizamos também a electroforese de duas dimensões para caracterizar algumas proteínas em particular que pareciam sofrer oxidação circadiana.

Após a quantificação das proteínas carboniladas, observou-se que os mesmos *spots* eram comuns em todos os tempos, mesmo se o nível de carbonilação é notavelmente diferente. Concluindo, nós provamos, embora sem proceder a uma caracterização final, que proteínas específicas possuem oxidação circadiana. No futuro, pode ser interessante identificar as proteínas alvo específicas e compará-las com os marcadores biológicos do stress oxidativo de forma a perceber melhor o envolvimento destas na ritmicidade circadiana.

Palavras Chave: Ciclo Circadiano; *Serum Shock* ; Dexametazona; Proteínas Carboniladas; Envelhecimento

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ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium sulfate
1D	One dimension
2D	Two dimension
APS	Ammonium peroxodisulfate
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
BMAL1	Brain and muscle ARNT-like 1
BSA	Bovine serum albumin
CKIε	Casein Kinase Iε
CLOCK	Circadian locomotor output cycles kaput
CO_2	Carbon Dioxide
CRY	Cryptochrome
Cy5	Cyanine 5
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenil
DNPH	2,4-dinitrophenylhydrozine
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECL	Enhance chemiluminescence method
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ET,1	Endothelin-1
EtBr	Ethidium Bromide
FGF	Fibroblast growth factor
G	L-Glutamine
GSK3	Glycogen Synthase Kinase3
h	hours
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HEK	Human Embrionary Kidney

KH_2PO_4	Potassium di-hydrogen phosphate
$MgCl_2$	Magnesium chloride
$Na_2HPO_4(H_2O)_7$	Disodium hydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
ºC	degrees Celsius
P	Penicilin-streptomycin
PAS	PER-ARNT-SIM
PBS	Phosphate buffered saline
PER	Period
PGE2	Prostaglandin 2
qPCR	quantitative Polymerase chain reaction
REV-ERBα	Reverse erythoblastosis virusα
RHT	Retinohipotalamic tract
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROR	Related orphan receptor
RORE	Related orphan receptor element
ROS	Reactive oxygen species
RT	Reverse transcription
S or FBS	Serum or Fetal Bovine Serum
SAD	Seasonal affective disorders
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
TTFL	Transcriptional-translational feedback loop

1. INTRODUCTION

1.1. Circadian Rhythms

All the organisms, from unicellular, like *Neurospora crassa* and cyanobacteria, to humans, show circadian rhythmicity in their biological, physiological and behavioural processes (Kondratov, 2007; Xydous, *et al*., 2012). The concept of circadian cycle was introduced by Halberg in 1959 and it comes from Latin "Circa Diem" which means "about a day" (Kondratov, 2007; Ramsey, *et al*., 2007). This concept is used to describe all the biological processes that exist in the organisms with a rhythmicity of 24 h (Kondratov, 2007; Ramsey, *et al*., 2007; Xydous, *et al*., 2012). There are other types of cycles with rhythmicity, however, those do not occur in intervals of 24 h. When a cycle has a rhythmicity bigger than 30 h, like the menstrual cycle in women, it is called infradian cycle and when something occurs in intervals lesser than 18 h, such as the case of cardiac frequency, it is called ultradian cycle (Sukumaran, *et al*., 2010; Yagita & Okamura, 2000). Lots of external (*Zeitgebers*) or internal (circadian clock) controllers are capable of controlling the circadian rhythmicity of a series of biological processes (Balsalobre, *et al*., 1998; Kondratov, 2007). Light is classified as the principal *Zeitgeber* which stimulates a series of molecular events in cascade which are capable of inducing rhythmicity in the expression of several genes (Bunney & Bunney, 2000; Fuller & Fuller, 2002). The chemical reagents and the social behaviour are some of the examples of the other controllers (Balsalobre, *et al*., 1998).

Important biological processes have a circadian rhythmicity as is the case of glucose, lipids and drug metabolism, proprieties of the membranes, division and cell cycle (promoted by *Cyclin B1*), blood pressure and many others (Balsalobre, *et al*., 1998; Gallego & Virshup, 2007; Kondratov, 2007; Weinert & Waterhouse, 2007). So, when a deregulation of the circadian cycle happens, lots of reactions and diseases can be induced (Froy, 2011; Gallego & Virshup, 2007; Kondratov, 2007; Reddy, *et al*., 2005). Many diseases and health problems exist due to the flaw of this cycle, like cardiovascular problems, cancer, hypertension or diabetes (Lévi, *et al*., 2010; Yu & Weaver, 2011).

Studies which have been made in shift workers, individuals subject to jet lag or people who live in northern latitudes show that these people have a deregulation of their circadian cycle which induces the appearance of many biological reactions (Albrecht & Eichele, 2003; Bunney & Bunney, 2000; Froy, 2011; Kondratov, 2007; Reddy, *et al*., 2005). In the first two cases the people show some sleep problems, fatigue, gastrointestinal problems and hypertension (Froy, 2011; Kondratov, 2007; Reddy, *et al*., 2005). However, in the last case the people have seasonal affective disorders

(SAD), also known as winter depression (Albrecht & Eichele, 2003; Bunney & Bunney, 2000). This syndrome was characterised as always appearing in the same period of the year, usually in winter, because the days are shorter and the solar intensity decreases (Bunney & Bunney, 2000). It must also be noted that persistent exposure to this type of work or life style can bring diseases like cancer (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Reddy, *et al*., 2005).

The circadian cycle in mammals is controlled by three different components: input pathway, the circadian master (Suprachiasmatic nucleous- SCN) and output pathway (Balsalobre, *et al*., 1998; Kondratov, 2007; Sukumaran, et al., 2010; Xydous, *et al*., 2012; Yagita & Okamura, 2000). This cycle has lots of specific proteins that control the physiological homeostasis which can influence all the tissues and cause some diseases related with aging (Kondratov, 2007; Weinert & Waterhouse, 2007).

1.2. Organisation of the circadian cycle in mammals

In mammals, the circadian system is organised in a hierarchical way (Kondratov, 2007). Anatomically and functionally, the circadian clock is characterised by having three main elements in its constitution: input pathway; pacemaker master and output pathway (Balsalobre, *et al*., 1998; Bunney & Bunney, 2000; Fuller & Fuller, 2002; King & Takahashi, 2000; Kondratov, 2007; Sukumaran, *et al*., 2010; Xydous, *et al*., 2012; Yagita & Okamura, 2000). The input pathway is classified as the one which is responsible for the transmission of the ambient conditions to a pacemaker master. On the other hand, the central master produces stimuli or signals by the output pathway for the whole organism and promotes the synchronization and the rhythmicity of the genes expression and the metabolic activities (Fu & Lee, 2003; King & Takahashi, 2000; Kondratov, 2007).

In 1972, Moore and Stephan, classified the suprachiasmatic nucleus (SCN) as the circadian master clock of the mammals (Froy, 2011; Kondratov, 2007; Kondratov, *et al*., 2003; Okamua, *et al*., 2010; Ramsey, *et al*., 2007). This central clock is located in the anterior hypotalamus, specifically in the 3rd ventricle, and it is composed of around 20000-100000 neurons (Fu & Lee, 2003; Fuller & Fuller, 2002; Kondratov, 2007; Reppert & Weaver, 2001). Although there are other controllers to command the SCN, light is considered the principal one (Balsalobre, *et al*., 2000; Balsalobre, *et al*., 1998; Kondratov, 2007; Lévi, *et al*., 2010). The photonic information is absorbed by the retina and it is sent by input pathway through the retinohipothalamic tract (RHT) to the SCN (Balsalobre, *et al*., 2000; Balsalobre, *et al*., 1998; Ramsey, *et al*., 2007; Sukumaran, *et al*., 2010).

This will then produce a response or signal (humoral or neuronal) that by output pathway goes to the peripheral clocks that exist in various tissues or organs of the organism promoting their synchronization (Albrecht & Eichele, 2003; Kondratov, 2007; Kondratov, *et al*., 2003; Okamua, *et al*., 2010; Ramsey, *et al*., 2007).

Taking into account the procedure of endogenous synchronization of the organism, the SCN is classified as being at the top of the hierarchical organisation (Figure 1), since this is what promotes the synchronization in all circadian oscillators located in the clocks of the peripheral tissues (Albrecht & Eichele, 2003; Kondratov, 2007; Kononenko, *et al*., 2008). Due to the importance of the SCN to synchronize the body, a deregulation of this pacemaker may promote multiple and serious health problems (Fuller & Fuller, 2002; Kondratov, 2007).

The circadian clocks that exist in the peripheral tissues in mammals answer, not only to the signalization promoted by SCN, but also to the multiple daily activities such as feeding, locomotion and others (Balsalobre, *et al*., 2000; Fu & Lee, 2003; Kondratov, 2007). In some situations they may act independently from the pacemaker, for example in case of damage of DNA. When this happens, they will control the local cell cycle checkpoints or they will turn on the apoptose (Fu & Lee, 2003). The similar circadian oscillators that were found in SCN exist not only in the peripheral tissues but also in the cellular culture (Froy, 2011; Gallego & Virshup, 2007). However, to promote a circadian rhythmicity of the clock genes expression in cellular culture, a first synchronization stimulus is necessary (Okamua, *et al*., 2010).

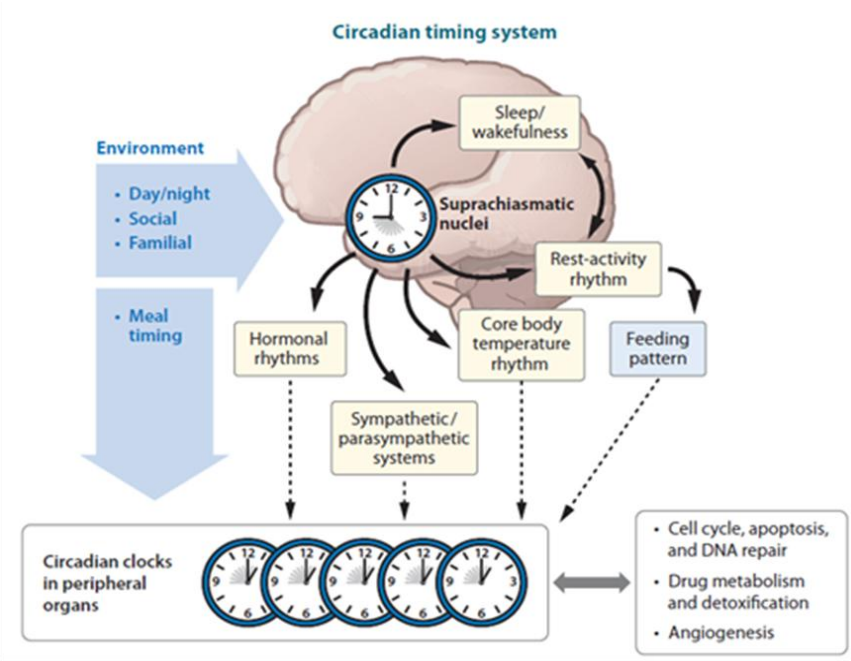

Figure 1- Schematic representation of the circadian cycle. The environmental active conditions, by input pathway, in the suprachiasmatic nucleus (SCN), which are the central pacemaker in mammals. This master promotes stimulis or signals that go, by output pathway, to the circadian clocks in peripheral organs and synchronizes lots of important biological processes (cell cycle, apoptosis, angiogenesis and others). However, the circadian clocks in peripheral organs can also be synchronized by daily activities like feeding. Adopted from (Lévi, *et al*., 2010).

1.3. Molecular mechanism of the mammalian circadian clock:

Molecular Biology is a science which has allowed the understanding of the behaviour and characteristics of the organisms through chemical mechanisms of activation and transcription genes. Lots of studies in molecular biology have been made to know the generation and control of the circadian rhythms, because they arise from the activation of expression and genes products (King & Takahashi, 2000; Okamua, *et al*., 2010). The circadian clock is characterised as being constituted by a set of specific genes, called by clock genes (Balsalobre, *et al*., 2000; King & Takahashi, 2000). These genes are characterised for working through the interaction between negative and positive transcriptional-translational feedback loops (TTFL) that promote the regulation of many biological activities (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Kondratov, 2007; Reppert & Weaver, 2001; Sukumaran, *et al*., 2010; Xydous, *et al*., 2012). A clock gene was defined as a necessary product to the regulation of the circadian clock, which may be expressed by circadian form (24 in 24 hours) or constantly during every day (Balsalobre, *et al*., 2000). In 1971 the first clock gene, *period* (*Per*), was identified in a study carried out in *Drosophilas melanogaster* (Balsalobre, *et al*., 2000; Fuller & Fuller, 2002; Ramsey, *et al*., 2007). In the 90s Joseph Takahashi identified the first clock gene in mammals, the transcription factor, *circadian locomotor output cycles kaput* (*Clock*). Nowadays 8 genes in the mammal's circadian clock which are homologous to the *Drosophila melanogaster* have already been identified (Albrecht & Eichele, 2003; King & Takahashi, 2000; Ramsey, *et al*., 2007).

The molecular mechanism of the circadian cycle in mammals is constituted by two feedback loops: a positive and a negative (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Kondratov, 2007; Okamua, *et al*., 2010; Reddy, *et al*., 2005; Reppert & Weaver, 2001; Sukumaran, *et al*., 2010). There are two principal components of the molecular mechanism in the circadian clock in mammals, an activator heterodimer which promotes the positive feedback loop and a repressor heterodimer, which is the responsible for the negative feedback loop (Gallego & Virshup, 2007; King & Takahashi, 2000; Kondratov, 2007; Okamua, *et al*., 2010). The activator heterodimer is constituted by two proteins: CLOCK and brain and muscle ARNT-like protein1 (BMAL1), also called MOP3. These proteins are transcription factors basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) that regulate the transcription of many clock genes (Albrecht & Eichele, 2003; Froy, 2011; Fuller & Fuller, 2002; King & Takahashi, 2000; Kondratov, 2007; Ramsey, *et al*., 2007; Reppert & Weaver, 2001; Sukumaran, *et al*., 2010).

When the CLOCK/BMAL1 complex binds to the E-box elements, especially in 5'-CACGTG-3' nucleotides sequence, it promotes the transcription of three kinds of genes *Period* (*Per1*, *Per2*

and *Per3*) and two types of genes *Cryptochrome* (*Cry1* and *Cry2*) (Figure 2). This mechanism has the name of positive feedback loop. And, due to this derivatization, proteins with the same name of genes are formed (Albrecht & Eichele, 2003; Froy, 2011; Fuller & Fuller, 2002; Gallego & Virshup, 2007; Ramsey, *et al*., 2007; Reppert & Weaver, 2001). PER and CRY proteins are formed in the cytoplasm by hyperphosphorylation promoted by Casein Kinase Iε (CKIε) and Glycogen Synthase Kinase-3 (GSK3) and when they are in the nucleus they form a PER/CRY complex (Albrecht & Eichele, 2003; Gallego & Virshup, 2007). This complex is called repressor complex and it is the responsible for the negative feedback loop, because it blocks the activity of the complex CLOCK/BAML1, so the transcription of clock genes is stopped (Albrecht & Eichele, 2003; Balsalobre, *et al*., 2000; Froy, 2011; Gallego & Virshup, 2007; Okamua, *et al*., 2010; Reppert & Weaver, 2001). The fact that the levels of *Per* and *Cry* genes decrease takes to the start of the cycle with the induction of positive feedback loop (Albrecht & Eichele, 2003; Gallego & Virshup, 2007; Reppert & Weaver, 2001). On the other hand, the increase of negative feedback limb promotes the positive feedback limb in processes that happen with a rhythmicity of 24 h (Reppert & Weaver, 2001). In diurnal animals, the positive feedback loop happens at the beginning of the day, with light period and the negative feedback loop happens more at the end of the day, with dark (Sukumaran, *et al*., 2010).

The positive feedback loop is also involved in a regulation of transcription of *Reverse Erythoblastosis Virusα* (*Rev-Erbα*) and *Related Orphan Receptor* (*ROR*) genes that control the expression of *Bmal1* gene (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Kondratov, 2007; Ramsey, *et al*., 2007; Sukumaran, et al., 2010). The *Rev-Erbα* gene can be regulated by other processes, like adipogenesis and carbohydrate metabolism. It is characterised as being the negative regulator of the expression of *Bmal1*, because it stops the transcription of this gene (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Ramsey, *et al*., 2007). On the other hand, ROR is defined to be the positive regulator, because via retinoic acid Related Orphan Receptor Response Element (RORE) it promotes the transcription of *Bmal1* (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Sukumaran, *et al*., 2010).

Studies which have been made over the years show that 10% of the total genes are influenced by the circadian cycle. However, some of them can be specific from some organs which underscore the importance of the regulation of the circadian cycle in life (Okamua, *et al*., 2010). Besides this, it was also observed that, between different species, the components of the circadian clock expression can be changed but the basic mechanisms are conserved (Gallego & Virshup, 2007).

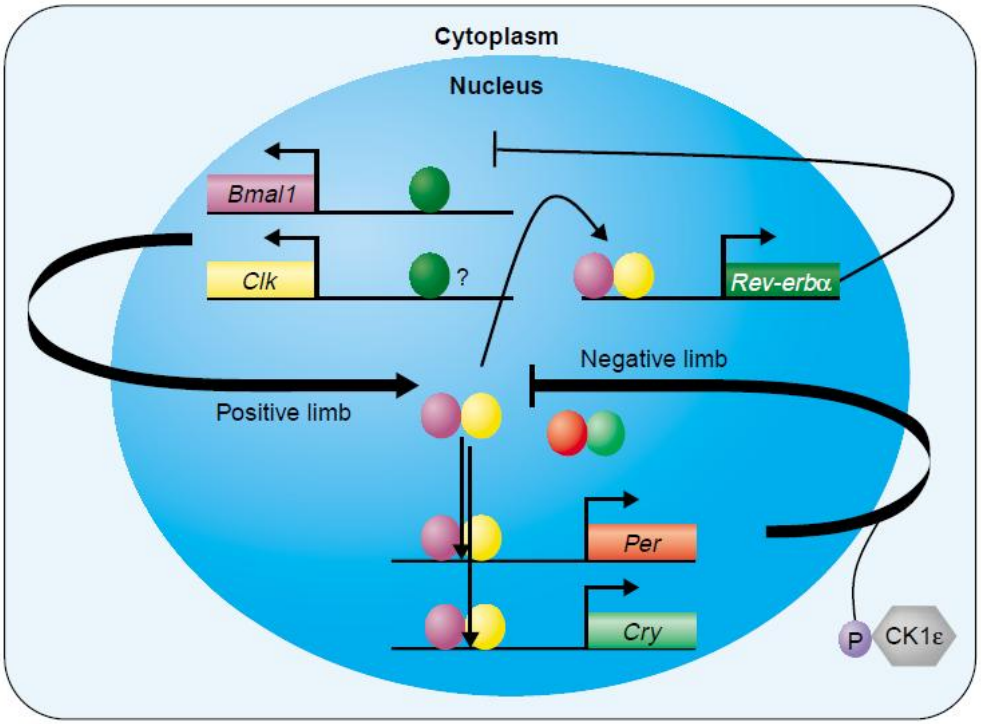

Figure 2- Representation of the molecular mechanism of the circadian clock in mammals. The positive limb happens when CLOCK and BMAL1 proteins make a heterodimer binding to the E-box elements promoting the transcription of many genes like *Period* (*Per1*, *Per2* and *Per3*), *Crypthocrhome* (*Cry1* and *Cry2*) and *Rev-Erbα*. These genes suffer hyperphosphorylation by Casein Kinase Iε (CKIε) and Glycogen Shyntase Kinase3 (GSK3) and form the correspondent proteins. The PER and CRY proteins bind and form a heterodimer called repressor heterodimer when they are inside the nucleus. This complex promotes the negative feedback loop because it stops the activity of the CLOCK/BMAL1 complex, so does the transcription of genes. The REV-ERBα, on the other hand it stops the expression of *Bmal1* gene at the transcription level. This feedback loop happens with a rhythmicity of 24 h and a disruption can bring lots of problems to the health of the organism. Adapted from (Albrecht & Eichele, 2003).

1.4. Metabolic processes with circadian cycles

Many studies have demonstrated that the expression and activity of all the circadian proteins of the circadian cycle are important, not only to genetic processes, but also to the homeostasis of many metabolic and cytosolic processes (Froy, 2011; Kondratov, 2007; Rey & Reddy, 2013; Sukumaran, *et al*., 2010; Tevy, *et al*. 2013). A disruption of transcription-translation feedback loop (TTFL) can promote lots of diseases at the metabolic level, degenerative or carcinogenesis which can influence aging (Rey & Reddy, 2013; Tevy, *et al*. 2013). However, recently it was observed in the cyanobacterium *Syncchococcus elongates* that, even in the absence of transcription-translation feedback loop, a biochemical oscillation catalysed by several clock proteins occurs (O'Neill, *et al*., 2011). This fact has crested the doubt on the TTFL being the only controller of the circadian cycle. Other studies which were made before in Eukaryotic systems indicated that the cytosolic metabolism is also involved in the controlling of the circadian rhythmicity (O'Neill, *et al*., 2011). In

another study made in 2011 by O'Neill and his collaborators it was observed that in the human blood cells there are cells without nucleus, so without TTFL, shows the circadian rhythmicity in the peroxiredoxin oxidation cycles. Peroxiredoxins are antioxidant proteins which allow to control the levels of intracellular peroxide because they have essential catalytic cysteine residues that use thioredoxin to remove this reactive species of oxygen (Eter, *et al*., 2013; O'Neill, *et al*., 2011). It can be concluded that the circadian rhythmicity could have an involvement in the regulation of ROS. Indeed, the ROS can serve as signals coupling metabolism to other cell functions (Gyongyosi, *et al*., 2013).

Moreover, it was demonstrated that although the nucleus was essential to sustain the circadian rhythmicity in mammal cells it was important, however, to understand the relationship between these two types of circadian controllers (O'Neill, *et al*., 2011).

1.5. Interaction between the transcriptional clock and the metabolic clock

The redox cycle (NADH/NADPH) was studied in human red blood cells and it was observed that it has a period of around 24 h. The oscillation of NADH/NADPH can be associated to the modulation of DNA-binding activity of the core circadian transcription factors of two clock genes, *Clock* and *Bmal1*. Besides this when the level of ATP are studied for 48 h it shows two cycles of circadian oscillations, which confirm the idea that the cycles observed in these cells have a metabolic origin (O'Neill, *et al*., 2011). The Ca^{2+} and cAMP seem, also, to be interrelated with the clock mechanism (O'Neill, *et al*., 2011; Rey, *et al*., 2013). In another work done by Eter and other scientists, they studied the relationship between the oxidation cycles of peroxiredoxin and diabetic type II with the peripheral atherosclerotic disease (PAD). The increase of peroxiredoxin is usually associated to this dysfunction and to the resistance to insulin and both are related to aging, but it was also related to a disruption of circadian cycle (Eter, *et al*., 2013). Nowadays it is very important to make a relation between the TTFL and cytosolic processes to study many diseases related to them (O'Neill, *et al*., 2011; Rey, *et al*., 2013; Tevy, *et al*., 2013). With all these observations nowadays the nuclear rhythms are associated to the cytosolic mechanisms (Figure 3), working like two motors of rhythmicity controlling several processes that are important to our health. Considering normal cells (cells with nucleus) it was proposed that the non-transcription oscillator and TTFL drive the peroxiredoxin rhythms and in cells without nucleus the peroxiredoxin rhythms are just driven by non-transcription oscillators (O'Neill, *et al*., 2011). A disruption of circadian

clock is associated with an increase of ROS and promoting of aging, but this finding can also be related with the oxidation cycle of peroxiredoxin or non-transcriptional oscillations.

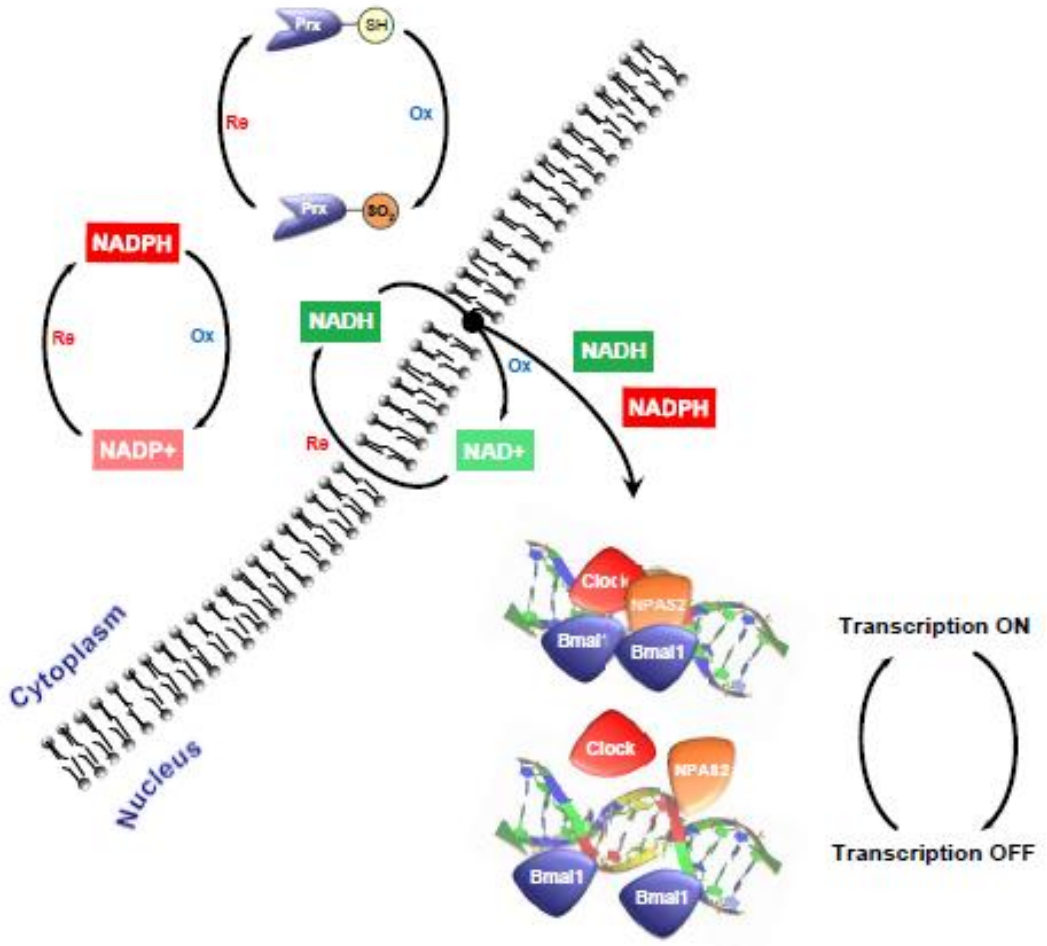

Figure 3- Propose of interconnectivity circadian redox processes and transcription-translation feedback loop (TTFL). Nowadays it is known that these two types of feedback work together to promote and control all organisms and like this control the homeostasis. The TTFL consists in a cycle and conjugation of positive and negative feedback loop. On the other hand it was discovered that the peroxiredoxin and the NADH/NADPH redox status has circadian rhythmicity and there is an interconnection between these two clocks. It was shown that NADH/NADPH redox status can directly modulate the DNA-binding activity of CLOCK/BMAL1 and control the TTFL. Adopted from (O'Neill & Reddy, 2011).

1.6. Circadian Cycle, Aging and Diseases

The circadian cycle is an important property for the good function of cellular and organism, so an anomaly in this cycle could cause serious health problems (Kononenko 2008; Lévi 2010). There are many diseases and health problems associated to dysfunctions of this cycle for example:

obesity, diabetes, degenerative diseases, aging, hypertension, cancer among others (Kondratov 2007; Lévi 2010; Yu 2011). The understanding of the molecular mechanisms of the circadian clock was the key to establish a relationship between the disruption of the circadian cycle and the development of some diseases or health problems (Kondratov, 2007; Sukumaran, *et al*., 2010). Many inflammatory diseases have symptoms that may be related to changes of this cycle, such as in the case of patients with a variation of rheumatoid arthritis who have more pains at night or in the early morning, or in the case of the patients with asthma, allergy, fever or rhinitis who have symptoms like nasal congestion, bronchial constriction and sneezing at night or when they wake up (Bechtold, *et al*., 2010). Besides this, it is possible to establish a framework of greater occurrence of cardiovascular events with periods of the day, as there are a greater number of episodes of stroke, myocardial infarction and sudden death during the morning period. This may be related to variation in blood pressure, heart rate and fibrinolysis activity that is directly dependent on the circadian cycle (Kondratov 2007).

The desynchronization can occur due to modifications of behaviour stimulus or because of changes in the expression and activity of the circadian proteins (Kondratov, 2007). Shift workers or people who are usually subject to jet lag are associated to the development of some health problems such as: insomnia, fatigue, gastrointestinal disorders, cardiovascular diseases, hypertension and diabetes, because the physiological processes and the normal hormonal state have changed (Albrecht & Eichele, 2003; Froy, 2011; Gallego & Virshup, 2007; Kondratov, 2007; Reddy, *et al*., 2005; Sukumaran, *et al*., 2010). Jet lag is the concept that is used to explain the trips made several times and for short periods of time, as the case of business people travelling from one continent to another. In these cases due to the time zone it is common for the people involved to have an "exchange of sleep", thus deregulating the circadian cycle (Bechtold, *et al*., 2010). The residents in countries of nordic latitudes are, also, subject to circadian dyshynchronization because in the winter the days are shorter and the 12 h cycles of light/darkness do not happen. Usually, these people develop a winter depression, also called by seasonal affective disorders (SAD) (Albrecht & Eichele, 2003; Bunney & Bunney, 2000).

The BMAL1 protein was considered as the principal element key to control the rhythmicity of the expression of the circadian genes (Kondratov, 2007; Sukumaran, *et al*., 2010). In 2006, Kondrakov and his collaborators verified that mice with a disruption of *Bmal1* had a predisposition to the development of diseases related with premature aging. When they compared these mice with wild type mice at the same age they saw that the mutant mice had signals of sarcopenia (decreased number of muscular fibres and its diameter), osteoporosis (reduced bone mass and its composition), cataracts, chronic inflammation of the cornea and alteration of the cell composition in the blood

(Kondratov, *et al*., 2006; Kondratov, 2007). Since the PER proteins (PER1 and PER2) are involved in the regulation of the transcription of *Bmal1*, the activity and expression of PERs and BMAL1 may be related (Kondratov, 2007; Okamua, *et al*., 2010). In one of the studies of Lee and his collaborators it was demonstrated that in mice with disruption of PER1 and PER2 a decrease of fertility was prematurely observed, when compared with wild type mice the same age (Kondratov, 2007). It was demonstrated that a disruption in one of these two proteins could affect important metabolic processes that exist in our body, including the development of cancer (Kunieda, *et al*., 2006; Okamua, *et al*., 2010; Sukumaran, *et al*., 2010). A disruption of BMAL1 promotes an increase of the level of reactive oxygen species (ROS) in the organism (Kondratov, 2007; Kunieda, *et al*., 2006; Myers & Badia, 1995). The BMAL1 protein is involved in the control of glucose metabolism, fat metabolism and homeostasis. These metabolic processes are the principal factors to control the levels of ROS and consequently the oxidative stress that is directly related with some age associated diseases (Kondratov, 2007; Ramsey, *et al*., 2007).

Aging can also promote some alterations in body composition and in the circadian cycle resulting in deregulation of homeostasis (Tevy, *et al*. 2013). This happens because some morphological, physiological and chemical changes in the circadian clocks occur with age (Fuller & Fuller, 2002; Myers & Badia, 1995). The principal variations in the cycle are: the decrease of amplitude, increase of phase cycle, alterations of body temperature, alteration of hormonal secretion and modification of sleep-wake cycle (Fuller & Fuller, 2002; Myers & Badia, 1995). In mammals it is known that the principal hormones which show circadian rhythmicity are leptin, insulin, and glucagon that can affect the blood glycemia and are related with premature aging. Indeed, some studies in humans showed that the increase of sensibility of our body to insulin can increase longevity. However, with aging the resistance to insulin increases together with abdominal obesity and sarcopenia (Tevy, *et al*. 2013). In conclusion, a deregulation in circadian clocks brings lots of problems to the health at the metabolic level and has been associated, also, with premature aging.

1.7. Aging and oxidized proteins

It was well described that the accumulation of oxidized proteins is a hallmark of cellular aging. The protein oxidation is caused by reactive oxygen species (ROS) mainly generated by mitochondria. However, when they are in excessive quantities they can promote oxidative stress which is directly associated with the development of many diseases related to aging such as amyotrophic dystrophy sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular

dystrophy, cataractogenesis, Werner's syndrome among others (Berlett & Stadtman, 1997; Butterfield & Kansi, 2001; Chondrogianni, *et al*., 2012; Dalle-Donne, *et al*., 2003). The ROS are inducing oxidative damage in many cellular components: DNA, carbohydrates, unsaturated lipids and proteins (Dalle-Donne, *et al*., 2003; Levine, 2002; Moller, *et al*., 2011). Proteins are among the most common target of oxidation (Dalle-Donne, *et al*., 2003; Moller, *et al*., 2011).

Proteins can suffer many types of damage, including the formation of carbonyl group (Chondrogianni, *et al*., 2012). Upon senescence of fibroblasts the oxidation of the proteins occurs essentially in four principal componentes: 44% in mitochondria, 28% in cytosol, 11% in endoplasmic reticulum and 8% in cytoskeletal. Carbonylation is one of the most common damage and is irreversible in proteins (Berlett & Stadtman, 1997; Cecarini, *et al*., 2007; Moller, *et al*., 2011). Consequently, the quantification of the level of carbonylated protein is one of the most used method to monitor the level of oxidized proteins (Chondrogianni, *et al*., 2012; Dalle-Donne, *et al*., 2003). Proteins can suffer two types of modifications when they are oxidized: first the alteration of side chains and second the cleavage of the peptide bond (Figure 4). The amino acids most susceptible to oxidative attack are: lysine, arginine, proline and theonine, leading to the formation of the carbonyl derivative proteins (Ahmed, *et al*., 2010; Chondrogianni, *et al*., 2012; Dalle-Donne, *et al*., 2003). The cellular homeostasis of this kind of protein damage is very important because the proteins have vital functions in many cellular processes (cellular synalisation, regulation of the cellular structure and enzymatic processes). Excess of the levels of the oxidized proteins has been associated with the development of many diseases (Berlett & Stadtman, 1997; Cecarini, *et al*., 2007). The mammal organism has developed mechanisms that control the accumulation of damaged proteins in order to maintain the cellular homeostasis, so when there is an increase in the level of oxidized proteins, they are eliminated by degradation or repair (Cecarini, *et al*., 2007; Chondrogianni, *et al*., 2012; Moller, *et al*., 2011). The oxidized proteins with reversible damage, like cysteine and methionine oxidation can be repaired by thioredoxin/thioredoxin reductase or glutaredoxin gluthation/gluthation reductase and methionine sulfoxide reductase, respectively (Ahmed, *et al*., 2010). On the other hand, the lysosome and proteosome are the two principal proteolytic systems responsible for the removal or degradation of the irreversibly damaged proteins (Ahmed, *et al*., 2010; Cecarini, *et al*., 2007; Chondrogianni, *et al*., 2012).

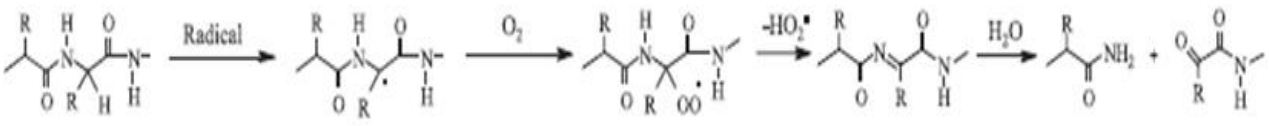

Figure 4 –Fragmentation of the carbon skeleton. The addition of the carbonyl group can lead to the modification of the carbon skeleton and/or to the modifications of the side chains (threonine, arginine, proline and lysine).

Usually, when the level of ROS increases, carbonylated proteins are formed, and there is a response to increase the proteasome's activity and the cellular homeostasis is kept (Butterfield & Kansi, 2001). Moreover, with aging there is a decrease of proteasome's activity and a decrease of regeneration of ATP, and both keep the increase of the level of ROS resulting in the accumulation of oxidized proteins (Ahmed, *et al.*, 2010; Chondrogianni, *et al.*, 2012). A decrease of the proteasome's activity related with aging is mainly due to the decrease in its cellular content, accumulation of its endogenous inibitors and oxidative and glycoxidative modifications in its subunits (Ahmed, *et al.*, 2010). Previous studies show that there is a relation between the circadian cycle and an increase of the level of ROS, essentially promoted by a disruption of circadian proteins, BMAL1 and PERs (Levine, 2002; Kondratov, 2007; Kunieda, *et al.*, 2006; Myers & Badia, 1995). Hence it is important to understand the relation between circadian changes and age-associated diseases related with oxidative stress (Figure 5) (Kondratov, 2007).

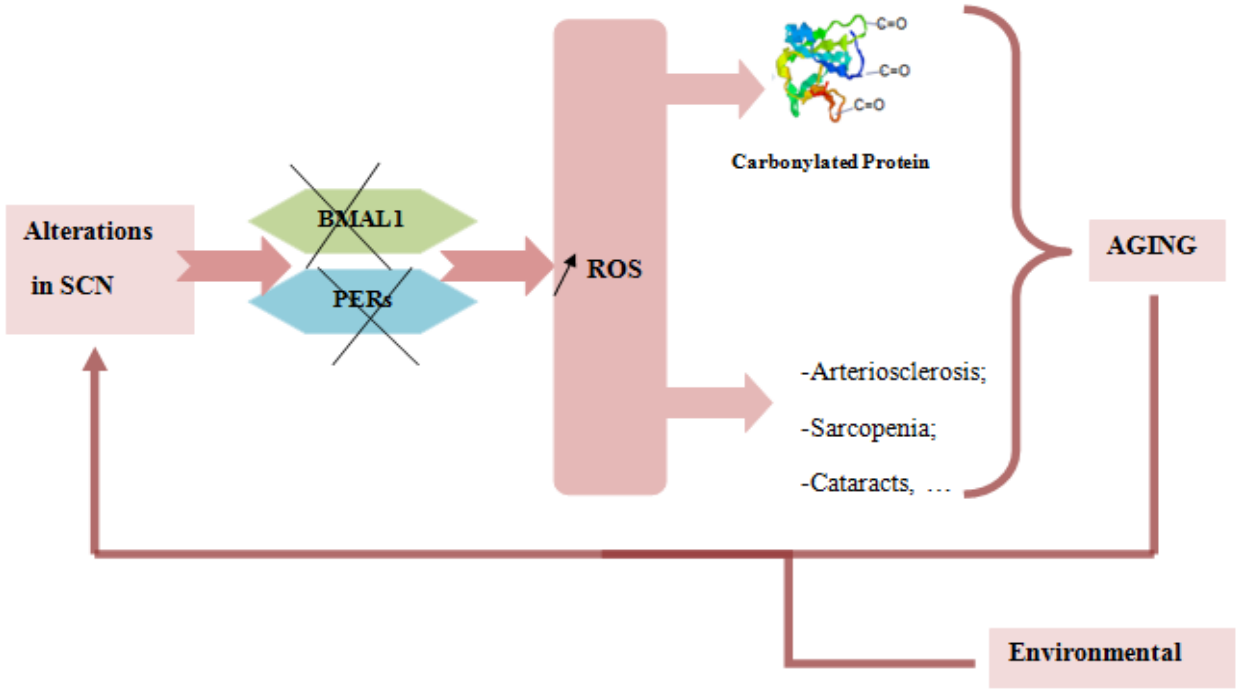

Figure 5- Relation between circadian rhythm aging and formation of carbonylated protein. When a change occurs in the SNC it may lead to a disruption of the protein BMAL1 and PERs and these are associated with an increase of the level of ROS and consequently an increase of the level of carbonylated proteins. Once the proteasome is unable to degrade all proteins which are damaged when there is a disruption accumulated as this will lead to aging. Furthermore, it is known that the increase of ROS is associated with the development of various diseases including cataracts, sarcopenia and arteriosclerosis that are associated with aging.

1.8. Circadian Synchronization in Cell Culture

Many studies associate aging or the development of some diseases related with age with a possible disruption in the circadian cycle. Thus it is becoming more and more interesting to understand how and why this relationship occurs (Fuller & Fuller, 2002; Kondratov, 2007; Kunieda, *et al*., 2006; Myers & Badia, 1995; Ramsey, *et al*., 2007). Nowadays it is known that the circadian clocks exist not only in the master pacemaker of the mammals, SCN, but also in the many peripheral tissues (Balsalobre, *et al*., 1998; Balsalobre, *et al*., 2000; Fu & Lee, 2003; Kondratov, 2007). The immortalized mammalian culture cells such as rat-1 fibroblasts and NIH3T3 are also capable of promoting circadian rhythmicity in genes expression *in vitro*, which was considered an asset for the studies in the circadian cycle (Balsalobre, *et al*., 1998; Balsalobre, *et al*., 2000; Izumo, *et al*., 2006). In mammals, the study of the circadian cycle is much more advantageous in culture cells than in cells of SCN. The culture cells are easier to sustain; can produce a large amount of material for biochemical assays and are easy access to molecular genetic tools. However, it is not possible to directly compare the studies performed in different cell types, because they can have different responses to the same treatment (Izumo, *et al*., 2006).

The circadian synchronization in cultured cells can be elaborated in many ways: Serum Shock, Dexamethasone, Forskolin (FSK), Epidermal growth factor (EGF) or Fibroblast growth factor (FGK), Calcium ionospheres, Glucose, Prostaglandin E2 (PGE2) and endothelin-1 (ET, 1). The first three ways are considered the most effective, depending, however, on the cell type in question (Izumo, *et al*., 2006). In this study HEK-293 cell was used and the best method to synchronize these cells, had to be chosen, between Serum Shock and Dexamethasone.

1.8.1. ***Circadian Synchronization with Serum Shock***

In 1998 Balsalobre and his collaborators performed a study in rat-1 fibroblasts. Initially they submitted the culture cells to higher levels of serum (50% of Serum in a usually cultured medium) for two hours and then put them in a medium without serum (Balsalobre, *et al*., 1998). After the treatment they used a medium without serum to avoid the cellular proliferation and placed all cells in the different phase of the cell cycle (Balsalobre, *et al*., 1998; Davis, *et al*., 2001). Through this experiment they verified that higher levels of serum induced the expression of *c-fos* and *Per* (*Per1*, *Per2*) genes, what is similar to what happened with light, which induced the expression of immediate early genes in SNC and has the same mechanisms (Figure 6). Besides this they observed

that the cultured cell kept the synchronization of the circadian genes in a medium without serum for 3 days (Balsalobre, *et al*., 1998). The use of this method to synchronize culture cells was carried out in many studies, which contributed to a better understanding of the circadian cycle and to what this can be associated with (Izumo, *et al*., 2006; Sukumaran, *et al*., 2010).

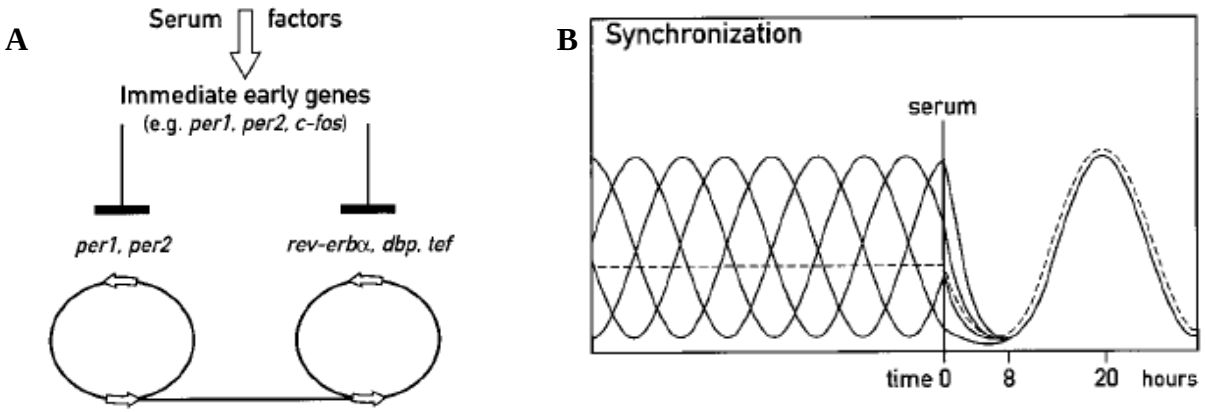

Figure 6- Possible explanation of synchronization with Serum Shock. **A-** When submitted by higher levels of serum the cells suffer an induction of immediate early genes (*c-fos*, *Per1* and P*er2)* due to some serum factors and with this, synchronized the transcription of the other circadian genes. **B**- When in the culture cell each one of the cells is in a different phase of circadian or cell cycle but when they are submitted by a serum shock treatment shows that they can be synchronized. However, it takes around 8 hours to have all the cells synchronized, so during this time it is possible to see a decrease of levels of the genes *per1* and *per2*. Adopted from (Balsalobre, *et al*., 1998).

1.8.2. ***Circadian Synchronization with Dexamethasone***

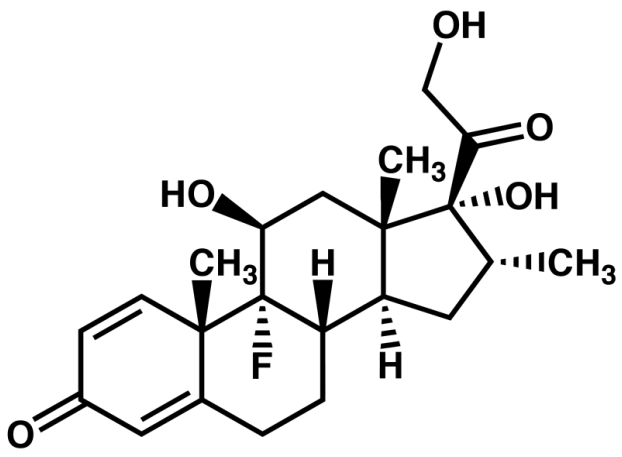

Figure 7- Chemical representation of dexamethasone (http://commons.wikimedia. org/wiki/File:Dexamethasone _structure.png)

The glucocorticoids are steroid hormones that the human body secretes in cycles and because of this they have been particularly attractive candidates for the circadian synchronization (Balsalobre, *et al*., 2000; Dickmeis & Foulkes, 2011). The dexamethasone (Figure 7) is a synthetically glucocorticoid that was used to promote the circadian synchronization in culture cell for the first time by Balsalobre and his collaborators in 2000 (Balsalobre, *et al*., 2000; Reddy, *et al*., 2005; Sukumaran, *et al*., 2010). These

investigators submitted rat-1 fibroblasts for 1 hour of treatment with dexamethasone and observed that the expression of *Per1* was increased very fast but the expression of *Per2*, contrarily to what happened with serum shock, didn't increase (Balsalobre, *et al*., 2000). The fact that dexamethasone is involved in many processes of the human body could be a reason for it to be a target form in therapy (Dickmeis & Foulkes, 2011). However, in some studies it is shown that this glucocorticoid can promote oxidative stress when it is used in a concentration higher than 10 mM. This can happen because dexamethasone increases the levels of one class of redox protein, Thioredoxin 2, and the increase of these is directly related to the increase of ROS leading the cell to suffer apoptose (Dickmeis & Foulkes, 2011; Psarra, *et al*., 2009). So, dexamethasone is considered one of the best methods of synchronization in some types of cells, however, it needs to be used in lower concentration so as not to promote oxidative stress and affect the circadian genes expression analysis (Psarra, *et al*., 2009).

2. OBJECTIVES

HEK-293 synchronized with serum shock as a cellular model studies of the laboratory showed that the level of carbonylated proteins have a circadian rhythm with a period of 24 h. It is known that these damages are directly related with the increase of ROS and that during aging there exist some specific proteins which are carbonylated and defined as biological markers of this process. In this view, to understand how the circadian cycle is implicated in the cellular aging, this work has the aims to:

Determine the variation of the level of carbonylated protein during the circadian cycle and identify the time when the maximum and minimum of carbonylated protein levels occur.

Optimize the 2D electrophoresis conditions in order to characterise some proteins that are preferentially targeted by the circadian oxidation.

3. MATERIAL AND METHODS

3.1. Culture of HEK-293 cells

To cultivate HEK-293 cells a classical medium, DMEM-PSG (Dulbecco's Modified Eagle Medium[1] (DMEM) supplemented with 1% of *Penicillin-streptomycin* (P), 10% of Fetal Bovine Serum (S or FBS) and 1% of L-Glutamine (G))*[2] were used. This medium contained all the nutrients which were needed and after the culture flasks were placed in an incubator chamber (CO_2 Incubator, MCO-17A1, SANYO) at 37°C, 5% of CO_2 and 95% of humidity. When the cells culture were made a laminar flow chamber (MSC Class II, TECHGEN), an aspirator Vacusafe comfort (IBS Integra BIOSCIENCES) and a microscope (CKx41, OLYMPUS) were used. Around 30 minutes before the procedure the necessary solutions were put in the water bath (JB Aqua 12 Plus, GRANT) at 37ºC and with this a temperature shock was avoided.

The total number of cells in suspension was counted by a NucleoCounter system (CHEMOMETEC) that counts the nucleus that exist in the suspension of mammalian cells previously treated with a Lysis Buffer and a Stabilizing Buffer. The propidium iodide which came inside the NucleoCassette operates as a dye, binds to the nucleus to facilitate its counting.

The cells were kept in pellet form for later analysis and for this it was necessary to make a centrifugation 5 minutes at 500 xg and 4°C (Allegra^{TM} X-12R centrifuge, BECKMAN COULTER).

3.2. Synchronization of the HEK-293 cells

When the cells in one of the flask T150 cm^3 were in confluence (around 80-90%), 2 million cells from other culture flasks were passed and were completed with the necessary quantity of classical medium. This type of flask was used to assure that enough material would be collected for all posterior analysis (qPCR, 1D and 2D electrophoresis). The cells were first cultivated in normal conditions which were described before (3.1.) and when they were in 80% confluence the synchronization was started.

At time zero (T=0 h) an addition of DMEM-PSG with the appropriate concentration of the synchronization reagent (serum and dexamethasone) was made. 2 h after, the medium was changed

[1] **D6546**- With 4500 mg/L glucose, sodium pyruvate, and sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture (SIGMA®-ALDRICH).
[2] (*) the composition is described in attachment 7.1.1.

to another one without serum (DMEM-PG)*[3] and the cells could start to be collected (usually in intervals of 4 h between each one). To know until which time the cells survived in a medium without serum, a control was made and in this way it was also proved that the non-synchronized cells do not have circadian cycles. For this the cells were cultivated as usual and it was considered time 2 h when the classical medium was changed by another one without serum, like in the other types of synchronization.

The HEK-293 cells were harvested without using trypsin because with only one wash with PBS 1X (SIGMA®-ALDRICH) they would peel off from the plastic. To put the appropriate quantity of cells for analysis they were counted and after they were centrifuged (from extraction of ribonucleic acid (RNA) around 2 million cells; for 1D electrophoresis around 4 million of cells and for 2D electrophoresis around 9 million of cells).

3.2.1. ***Synchronization with dexamethasone***

To make synchronization with dexamethasone (SIGMA®-ALDRICH) a final concentration of 10 nM of this reagent was used. A stock solution of dexamethasone of 10 mM was prepared, diluted in absolute ethanol and conserved at -80ºC. Then, two dilutions were made, the first one of 1:100 with phosphate buffered saline (PBS) 1X (SIGMA®-ALDRICH) and the second one with a reason of 1:1000 with complete medium. After this a common procedure of synchronization was made, as was described before (3.2.) until 50 h were completed.

3.2.2. ***Synchronization with serum shock***

A synchronization with serum shock in HEK-293 cells consists in preparing a culture medium with a higher concentration of FBS (DMEM-PSG and FBS in a dilution 1:2 for each flask) and the usual procedure for synchronization was made, as was described before (3.2.) until 50 h were completed.

[3] (*) the composition is described in attachment 7.1.2.

3.3. Analysis of the transcripts

A series of procedures were necessary to carry on with the analysis of the transcripts. The extraction of RNA samples and their quantification was performed in order to understand whether there was sufficient RNA to perform the analysis. Subsequently a reverse transcription (RT) was prepared so as to obtain deoxyribonucleic acid (DNA) *primer* sequences to be analysed by quantitative polymerase chain reaction (qPCR).

3.3.1. ***Extraction and quantification of RNA***

The extraction of RNA of the cells harvested at different times of synchronization was executed with a NucleoSpin® RNA II kit (MACHEREY-NAGEL). The centrifugation was made in a 1-15K, SIGMA®-ALDRICH and the quantity of RNA was determined with a NanoVue (GE HEALTHCARE).

To confirm the integrity of RNA an electrophoresis was performed. First, a gel with 1% of agarose (SIGMA®-ALDRICH) in 100 mL of solution TAE 1X*[4] was dissolved and after 2 µL of Ethidium Bromide (EtBr, SIGMA®-ALDRICH) was added. When the gel was ready and fixed, the mix of 5 µL of sample and 2 µL of solution DNA Dye 6X*[5] was put in each and the migration with a Power Suply-Model 250/2,5 (BIORAD) was started. For the RNA to be considered good, 2 bands in gel were needed, one that corresponded to RNAr 28S (4718 pb) and the other one to RNAr 18S (1900 pb).

3.3.2. ***Reverse Transcription***

The reverse transcription was done with a kit called SuperScript® III First – Strand Synthesis System for RT-PCR (Invitrogen, LIFE TECHNOLOGIES) which was designed to convert 1 µg of RNA to 5µg of total cDNA. To do this procedure 1µg of RNA was used in a final volume of 8 µL. In each tube 1 µL of Random Hexamers (50 mg/µL) and 1 µL of dNTP mix (10 mM) was added and after an incubation in a (Master Cycles Personal, EPPENDORF) for 5 minutes at 65ºC and 1 minute in ice was made. During this time, the solution DNAmix*[6] was prepared and in each sample

[4] (*) the composition is described in attachment 7.2.2.
[5] (*) the composition is described in attachment 7.2.3.
[6] (*) the composition is described in attachment 7.2.4.

10 µL of this solution was added and they were incubated in 3 steps. The first one corresponded to 10 minutes at 25ºC; the second one to 50 minutes at 50ºC and the last one to 5 minutes at 85ºC. When the incubation was finished it was necessary to add 1 µL of RNase H (*E.coli*) and the last incubation for 20 minutes at 37ºC was made so that after the samples were frozen for further studies.

3.3.3. ***Quantitative PCR***

Before the qPCR was made, there was a need to prepare a dilution 1:10 of the samples previously subject to extraction of RNA and reverse transcription (2 µL of sample subject to reverse transcription in 18 µL of RNA-free water) and the preparation of the standard curve*[7] and *primers* solution*[8]. In one specific plate for qPCR (Frame Star480, 4ATITUDE) 2 µL of each sample and 8 µL of the *primers* solution (Table 1) that was to be examined was added. A standard curve with dilution 1:5, 1:10, 1:20, 1:50 and 1:100 was prepared before. The dilution 1:5 was made with 10 µL of 3 different cDNA in a final volume of 150 µL.

The *primer* that was used for the reference was GAPDH because it did not have circadian cycles and the program of PCR consisted of four steps: denaturation, amplification, melting and cooling and it was made in Light Cycler480® Real Time PCR Systems (ROCHE). The denaturation was constituted by 1 cycle of 5 minutes at 95ºC. The amplification was founded by 43 cycles divided in 3 parts during 15 seconds each part at 95º, 60º and 72ºC, respectively. The melting step was created with just one cycle with 30 seconds at 95ºC, 30 seconds at 60ºC and after continuously until 95ºC. The program was finished with a last cycle of 30 seconds at 40ºC.

Table 1 – *Primers* of clock controlled transcripts for qPCR. All the primers were designed by Nicolas Ugarte with a programme AmplifX with the exception of *Cyclin B1* that was designed by Audrey Desvergne.

Primer	Forward	Reverse
GAPDH	5’ GTCATCCATGACAACTTTGGTATCG 3’	5’ GCAGGTCAGGTCCACCACTG 3’
Clock	5’ TTGCTCCATGGGAATCCCTCAACT 3’	5’ CGTGTGCTACTGTGGTTGAACCTT 3’
Bmal1	5’ AGCCCATTGAACATCACGAGTACG 3’	5’ AGTAGGCCACTGGAAGGAATGTCT 3’
Per2	5’ GGTGCACAGCCCTCATTCTTTTCA 3’	5’ CCTCACTTTTCCCCAAGTGTCCAA 3’
Cry1	5’ TGGGAATGGAGGCTTCATGGGATA 3’	5’ CCTTGCTTCAACAGGTGAGTTTGC 3’
Rev-erbα	5’ TGTTCAACGTGAAGGACCAGACAG 3’	5’ AAGTCGAACATGGCACTGAGCA 3’
Cyclin B1	5’ GAAGAGCCAGCCTAGCCTCAG 3’	5’ AATAAGGAGGGAGCAGTGCG 3’

[7] (*) the composition is described in attachment 7.2.5.
[8] (*) the composition is described in attachment 7.2.6.

3.4. Analysis of Carbonylated Proteins

To make an analysis of the proteins, an extraction and quantification was necessary. Initially a 1D electrophoresis was prepared to see the levels of carbonylated proteins along the circadian rhythmicity and after two time points, a maximum and minimum, were chosen to make a 2D electrophoresis. We analysed the level of carbonylated protein (Oxy ratio) when we made a ratio between the % Volume of Carbonylated Protein protein and the % Volume of total protein that exists in the sample.

$$\frac{\text{\% Volume of Carbonylated Protein}}{\text{\% Volume of Total Protein}} = \text{Oxy ratio}$$

3.4.1. ***Extraction and quantification of Protein***

The extraction of protein started with adding 350 µL of Lysid Buffer I*[9] for, around 10^7 cells. The samples were kept in ice for 15 minutes but were taken to vortex every 5 minutes or 15 minutes or more in Thermomix®Comfort for eppendorf of 1,5 mL (EPPENDORF) at 1400 rpm and 4ºC until they did not present viscosity. After, a centrifugation was made during 15 minutes at 10000xg at 4ºC. We quantified the protein that was in the supernatant with the Bradford Method (Bradford, 1976) using the Protein Kit Assay (BIORAD) and the Power Wave XS (BIOTEK). The rest of the samples were frozen at -80ºC. For the 2D electrophoresis we tested two types of Lysis Buffer I and II*[10], but for 1D electrophoresis we just used the Lysis Buffer I*[11].

For the quantification of protein by the Bradford Method a plate with 200 µL of final volume (5 µL sample or standart curve with 5 µL of MilliQ water or Lysis Buffer, respectively, and 190 µL of Bradford reagent) was prepared. The standard curve was made with different concentrations of bovine serum albumin (BSA) (0; 0,2; 0,4; 0,6; 0,8; 1).

3.4.2. ***One Dimension Electrophoresis (Oxyblot 1D)***

For each sample 10 µg of proteins were prepared with MilliQ water in a final volume of 4 µL. After, 6 µL of (SDS 10% from BIORAD) was added to denature the proteins. The OxyBlot Protein

[9] (*) the composition is described in attachment 7.3.1.1.
[10] (*) the composition is described in attachment 7.3.1.1. and 7.3.1.2.
[11] (*) the composition is described in attachment 7.3.1.1.

Oxidation Detection Kit (MILLIPORE) was used to start the derivatization (Figure 8, A). For this we used the 2,4-dinitrophenylhydrazine (DNPH). The protein was separated by SDS-PAGE (Figure 9, A) electrophoresis with a 12% polyacrylamide gel*[12] in a Migration Buffer*[13]. In each well 14 µL of the samples were added. In one of them we put 7,5 µL of the marker of molecular weight (Precision Plus Protein™ Standard, BIORAD). The electrophoresis was programmed to make 2 steps (100 V for 30 minutes; 200 V for 50 minutes) with the Power PAC HC™ (BIORAD).

The carbonylated proteins were detected by immunoblotting after being transferred to a nitrocellulose membrane. The support "sandwich" (BIORAD) was prepared inside the Tranfer Buffer*[14] by placing their components in the following order: sponge, Wattman paper (Amersham™ Hybond™ blotting paper, GE HEALTHCARE), gel, nitrocellulose membrane (Hybond™C Extra Amersham, BIOSCIENCES), Wattman paper and sponge (Figure 9, B). The transfer was made by Power PAC HC™ (BIORAD) which was programmed for 1 step of 100 V for 1 h. To see the total protein, the membrane was removed and placed in Fast Green solution*[15] (60 mL of ethanol absolute, 20 mL of acid acetic, 120 mL of MilliQ water and 0,02 g of Fast Green) for 5 minutes and after was revealed in Odyssey programme (Odyssey3.0V, LI COR). Odyssey Buffer*[16] was added to the membrane to make a blockage all night at 4ºC. Then, the membrane was incubated with 2 antibodies. The first one from the OxyBlot Protein Oxidation Detection Kit (MILLIPORE) which was an antibody against 2,4-dinitrophenyl, with a dilution 1:5000 in Odyssey Buffer (Figure 8, B). The second one was a Goat anti-rabbit IRDye®800CM from LI COR with a dilution 1:15000 in Odyssey Buffer. This incubation was made during 45 minutes but with 3 washes with Washing Buffer*[17] during 10 minutes each one between each antibody. Finally a new revelation was made in Odyssey programme.

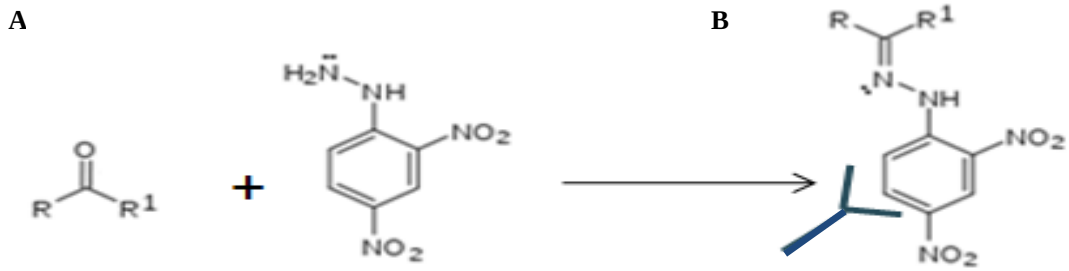

Figure 8-Scheme of derivatization with 2,4-Dinitrophenylhydrozine (DNPH) and reaction of the protein with the antibody anti-DNP. The DNPH binds to the protein and when the anti-DNP is added they form a protein DNP adduct.

[12] (*) the composition is described in attachment 7.3.2.
[13] (*) the composition is described in attachment 7.3.3.
[14] (*) the composition is described in attachment 7.3.4.
[15] (*) the composition is described in attachment 7.3.7.
[16] (*) the composition is described in attachment 7.3.8.1.
[17] (*) the composition is described in attachment 7.3.6.

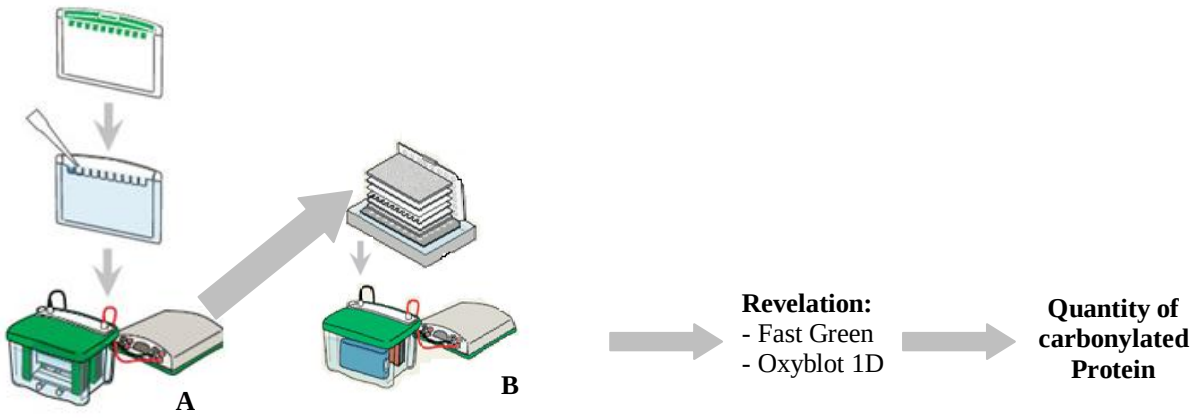

Figure 9- Representation of the method of 1D electrophoresis (Adapted from http://www.bio-rad.com).

3.4.3. *Two Dimension Electrophoresis (Oxyblot 2D)*

It was necessary to adjust the Oxyblot 2D procedure. In a first step a definition of quantity of protein was tested with Enhanced chemiluminescence (ECL) method. The Lysis Buffer I and II*[18] was also compared to two different strips (11 and 13 cm). For Odyssey revelation the second antibody, the anti-rabbit (LI COR) in a dilution of 1:5000 with Odyssey buffer*[19] was used, for ECL the anti-rabbit GtxRb by Oxyblot™ Protein Oxidation kit (MILILLIPORE) in a dilution 1:500 with BSA Buffer*[20] was chosen. To have a first idea and make a quantification of carbonylated proteins, we prepared an electrophoresis 2D in a small gel, Criterion™ TGX Any kD™ Precast Gel. We used this type of gel, because it provides the separation from 10-250 kD with the best resolution of the proteins in the 20-100 kD molecular weight range.

The Re-hydratation Buffer*[21] was prepared and the necessary quantity to make a solution with 300 µg of protein in a final volume of 250 µL, for 2D electrophoresis in a big gel or 100 µg of protein in a final volume of 200 µL for a 2D electrophoresis in a small gel, was calculated. This solution was added in each strip (Immobiline™ DryStrip pH 3-10NL, GE HEALTHCARE) and after 500 µL of mineral oil (GE HEALTHCARE) was also placed and this stayed in incubation all night at room temperature without light. When this stage was finished the EttanIPGphor3 with 150 mL of mineral oil was prepared and in each well the strip was put with a Paper Wicks on each side, previously humidified with 100 µL of MilliQ water with Destreak (12 µL of Destreak for 1 mL) and the electrodes were added after. All the material used in this step of focalisation came from GE HEALTHCARE. The focalisation was programmed with 4 phases that had a different voltage and

[18] (*) the composition is described in attachment 7.3.1.
[19] (*) the composition is described in attachment 7.3.8.
[20] (*) the composition is described in attachment 7.3.8.2.
[21] (*) the composition is described in attachment 7.3.9.

duration, depending on the type of strip that was used. For a strip with 13 cm (2D electrophoresis in a big gel): first Step at 150 V around 10 h; second Grad with 1000 V for 3 h; third Grad with 8000 V for 2 h and finally fourth Step with 8000 V for 1 h was made. For a strip with 11 cm (2D electrophoresis in a small gel): first Step at 500 V for 3 h; second Grad at 1000 V for 1 h; third Grad at 6000 V for 2 h and finally fourth Step at 6000 V for 40 minutes. When this was finished the strips were frozen inside a plastic.

The strips that were used for the oxyblot 2D we subjected to derivatization process while the strips for Coomassie Blue just needed to be equilibrated. The derivatization process consisted in 3 steps: first the strips with 10 mL of Derivatization Solution*[22] were placed for 20 minutes; second changed the strips to 10 mL of Neutralization Solution*[23] for 10 minutes and finally the strips were put again in 10 mL neutralization solution but for 30 minutes. The strips were placed 15 minutes in solution B1*[24] and after 15 minutes in solution B2*[25]. Depending on the strips that are used the duration of the migration and the transfer are different. A migration with 2 steps, one with 25 V and duration of 3 h and the other with 90 V and duration of 20 h was used for strips with 13 cm and a migration with 3 steps, one with 100 V for 20 minutes, another with 150 V for 50 minutes and a last one with 200 V for 15 minutes was used for strips with 11 cm. For the 13 cm strip we made a 12% acrylamide gel*[26] and for the 11 cm strip we used a precast gel Criterion™ TGX Any kD™ Precast Gel. The migration just started when the strips and the standard protein (Precision Plus Protein™ Standart, BIORAD) were closed to the gel and stuck with agarose (0,5g agarose and 100 mL of migration buffer 1X*[27]).

Then there was the transfer of protein to a membrane like in oxyblot 1D (made a "sandwich" and the things were placed in this order: sponge, Wattman paper, gel, nitrocellulose membrane, Wattman paper and sponge) but it took more time (3 h at 100 V) for the strips with 13 cm. It is important that the box to make a transfer was turned on to a refrigerator, in this case (MultiTempIII Pharmacia, BIOTECH). Later, the membrane stayed in Fast Green solution*[28] for 10 minutes to make a revelation of the total of proteins in Odyssey programme. After the membrane was washed 3 times (15 minutes each time), an incubation was made for one hour in slow agitation at room temperature with Blocking Buffer*[29] and the first antibody anti-DNP rabbit (SIGMA®-ALDRICH) was incubated with slow agitation for all night at 4ºC.

[22] (*) the composition is described in attachment 7.3.10.
[23] (*) the composition is described in attachment 7.3.11.
[24] (*) the composition is described in attachment 7.3.12.1.
[25] (*) the composition is described in attachment 7.3.12.2.
[26] (*) the composition is described in attachment 7.3.13.
[27] (*) the composition is described in attachment 7.3.3.
[28] (*) the composition is described in attachment 7.3.7.
[29] (*) the composition is described in attachment 7.3.8.

The second antibody was placed in contact with the membrane for 1 h at room temperature. It is important not to forget that the second antibody is fluorescent so the membrane was kept in the dark. Between each incubation there was a need of washing 3 times, 15 minutes each time with 100 mL of Washing Buffer*[30]. A revelation of membrane was made and after with 2D Platinium programme there was a detection and quantification of the carbonylated protein for the spots of interest to be identified by mass spectrometry.

3.4.3.1. Preparation and treatment of Coomassie gel

To make identification by mass spectrometry a gel with Coomassie was prepared to take the proteins/spots which were chosen. The strip for Coommasie gel was subjected to the same procedure as the others to make a membrane. However, it did not need to be subjected to derivatization because with this gel all the proteins existent in the sample in question were supposed to be seen and not only the carbonylated proteins. After the migration of the strip, the gel was subject to a wash of 30 minutes with 250 mL of MilliQ water, 2 washes of 30 minutes each one with 250 mL of Fixation Buffer*[31] and incubation all night with the last solution used with slow agitation in the cold room. On the other day there were three incubations of 10 minutes each one with a solution with 2% of acid ortophosphoric. After, an equilibration of 30 minutes with 250 mL of Pre-coloration Buffer*[32] and an incubation of 24 h to 72 h with Coloration Buffer*[33] was made. When this interval ended the gel was washed with MilliQ water and a scan was made. The gel was then kept in the fridge with pre coloration buffer until the spots were removed and only then was it sent to make a spectrometric mass analysis.

[30] (*) the composition is described in attachment 7.3.6.
[31] (*) the composition is described in attachment 7.3.14.
[32] (*) the composition is described in attachment 7.3.15.
[33] (*) the composition is described in attachment 7.3.16.

4. RESULTS

In this work three types of treatment in HEK-293 cells were tested. The first treatment consisted in a standard (WS) and the two other consisted in two different types of circadian synchronizations (synchronization with 10 nM dexamethasone-SD and synchronization with 50% of bovine serum-SSS), to see which was the best one for this type of cells. The preliminary results of A. Desvergne, which showed a circadian variation of the level of carbonylated proteins in HEK-293 cells synchronized by serum shock, were taken in account. The aim of this work was to see if some carbonylated proteins exhibited a circadian oxidation. For this purpose different types of analysis were made, like observing the cells during each treatment, making a qPCR in 5 clock genes to see the circadian expression of theme and quantifying the level of carbonylated proteins in 1D and 2D electrophoresis.

4.1. Determination of the best type of synchronization for HEK-293 cells

Microscopic analysis in HEK-293 cells was used during each treatment to compare the different stress effects of the three types of treatments which were submitted. In figure 10 pictures of the cells in four different times of each experiment can be seen: at the beginning of the treatment, considered time 0 h (A, E and I); 26 h (B, F and J); 34 h (C, G and K) and 46 h after the beginning of the treatment (D, H and L). Through this type of analysis the behaviour of the cells during each treatment could be compared. Pictures A, B, C and D correspond to standard cells (without synchronization-WS), in other words, cells that are submitted to deprivation of medium at the same time as the other cells that suffer synchronization. Around 26 h after the beginning of the experiment (B) it was observed that the cells began to have signs of stress. These cells are considered in stress when they have more elongated morphology and when some cellular islets are observed. In pictures C and D, 34 h and 46 h after the beginning of the treatment it could be observed that the cells had an increase of this stress morphology. Pictures E, F, G and H are subject to cells that suffered a treatment of 2 h in a classical medium supplemented with 10 nM of dexamethasone (synchronization with dexamethasone- SD) and it is obvious that during this treatment the cells also showed a stress behaviour, which seems to be more than in standard cells. Pictures I, J, K and L correspond to the cells at 0 h, 26 h, 34 h and 46 h submitted to a synchronization of 50% of bovine serum (synchronization with serum shock- SSS). Around 26 h

after the beginning of this treatment (J) the cells showed a light behaviour of stress, however 34 h (K) and 46 h (L) after, they showed a better state.

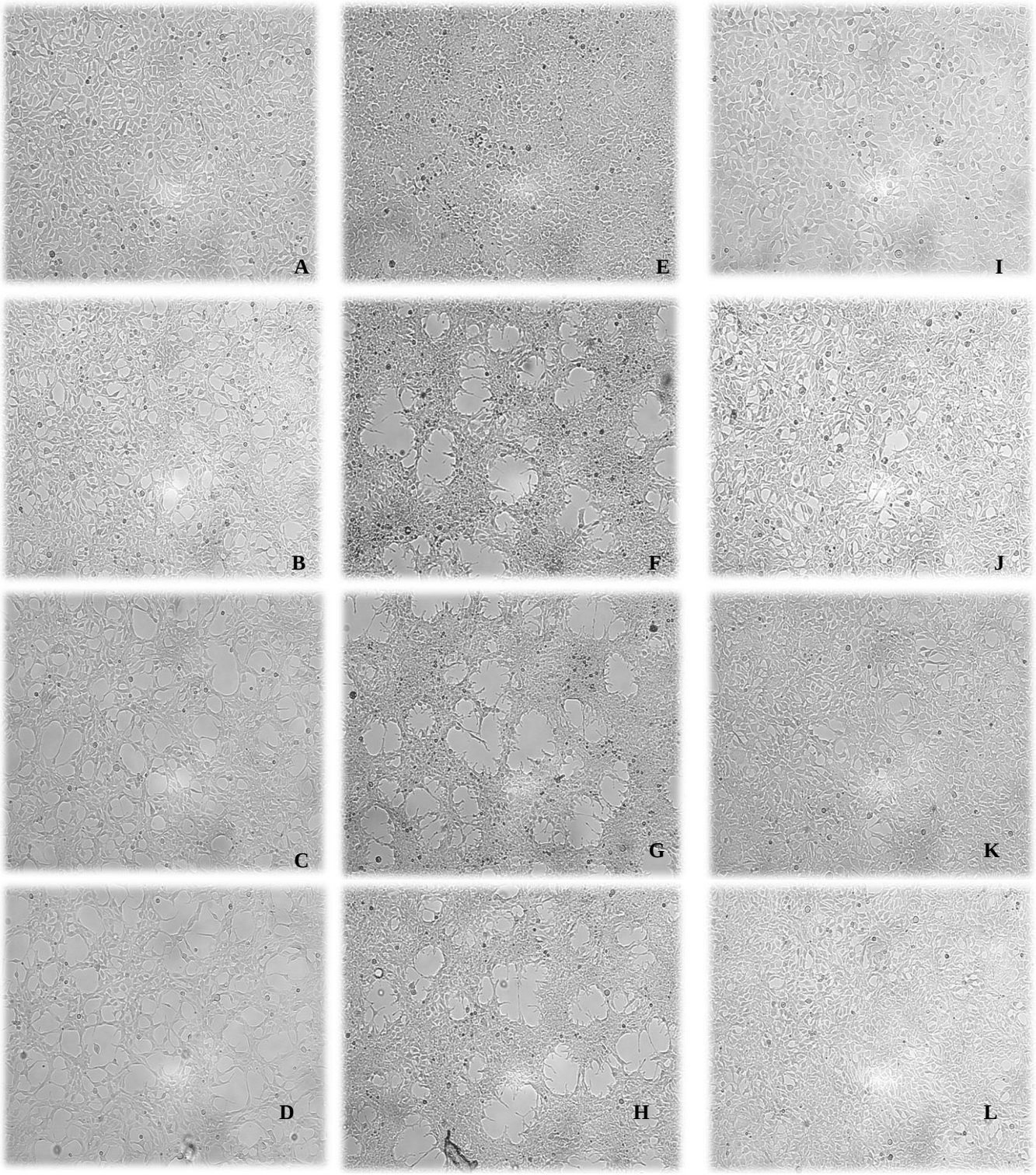

Figure 10- Microscopic analysis of HEK-293 cells in three types of treatment. Pictures A, B, C and D correspond to the cells in the treatment without synchronization (WS). Pictures E, F, G and H are of the synchronization with dexamethasone (SD) and pictures I, J, K and L are of the synchronization with serum shock (SSS). **A-** time zero, WS; **B**- 26 h, WS; **C**- 34 h, WS; **D**- 46 h, WS; **E**- time zero, SD; **F**- 26 h, SD; **G**- 34 h, SD; **H**- 46 h, SD; **I-** time zero, SSS; **J**- 26 h, SSS; **K**- 34 h SSS; **L**- 46 h, SSS. Objectives 10X.

Comparing the effects of the three types of treatment in HEK-293 cells it was evident that a simple deprivation of serum or the dexamethasone treatment had a higher stress effects in cells than with serum shock. The cells were subjected to serum deprevation after the treatments to assure that all the cells were not at the same phase of cell cycle and like this the division of cells was more slowly and less new cells non synchronized were collected during the experiment. Looking at the pictures it can be seen that the synchronization with serum shock seems to be the better for these types of cells suggesting that they suffer an adaptation to serum deprivation better than the other cells treated with dexamethasone. These early results led us to consider that the synchronization with serum shock would be more appropriate for our analysis. Indeed, the cells were closer to the normal morphology and submitted to a lower stress, what would be better for the carbonylated protein analysis. The validation of this type of synchronization was then performed by the analysis of the transcripts clock genes.

4.2. Quantitative PCR

The execution of a qPCR in our samples, which were collected during each treatment, allowed us the analysis of the clock genes expression. In this case the clock genes we tested: *Clock*, *Bmal1*, *Per2*, *Cry1* and *Rev-Erbα* to see if they were expressed with a circadian rhythmicity and to determine the precise period of this type of cells.

In order to confirm that the treatment with serum shock is the only responsible of the synchronization unsynchronized cells (left in serum free) were compared with synchronized cells (Figure 11). For this the transcript level of two clock gene *Clock* and *Per2* were compared. And we can observe that in Figures A and B related to the unsynchronized cells we don't have a circadian oscillation of the two clock genes contrary to what is observed in the graphics C and D where the cells were synchronized. In fact, two circadian cycles in each transcript can be seen. However, the start of the synchronization just occured 8-10 h after the start of the treatment, because according to Balsalobre, this is the time that the cells need to be synchronized. These analyses were used to determine the dominant period in the circadian field thanks to a spectral analysis program using the Fourier transform. And we found that for HEK cells we have a period included between 20 h and 27 h. In picture C, which is related to the level of *Clock* transcript, shows that it has a period of 27 h, what comproves the circadian rhythmicity that this treatment improves in the cells. In picture D that the period of the level of *Per2* transcript is 22,6 h.

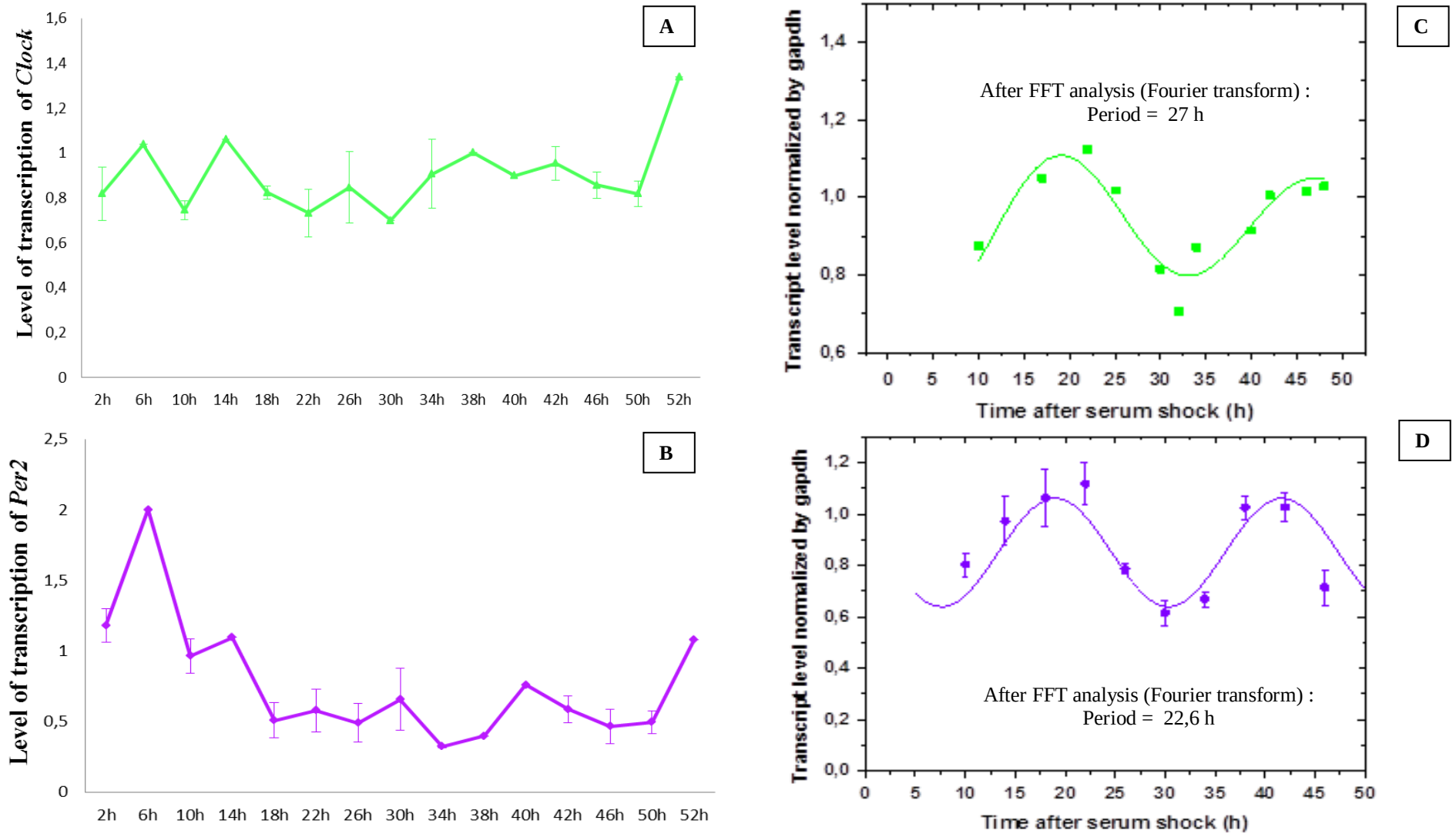

Figure 11- Transcription level of *Clock* and *Per2* normalized by GAPDH. Pictures A and B refer to the cells unsynchronization (WS), pictures C and D are relative to the cells synchronized by serum shock (SSS). **A-** Transcription level of *Clock* in cells WS; **B-**Transcription level of *Per2* in cells WS; **C-** Transcription level of *Clock* in cells SSS; **D-** Transcription level of *Per2* in cells SSS.

Then, to confirm that the serum starvation can inhibit the cell cycle, the transcript expression of a cell cycle marker was analysed: *Cyclin B1* (Figure 12). After an analysis by the Fourier transform it was seen that the period of this gene in synchronized cells is of 52 h which is totally different from the circadian period.

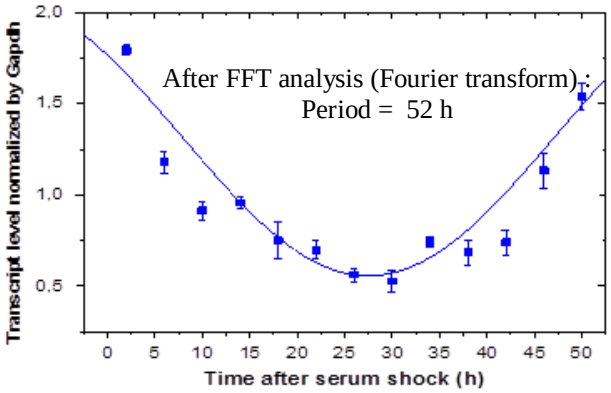

Figure 12- Transcription level of *Cyclin B1* in cells submitted to serum shock treatment.

In order to see if the carbonylated protein are submitted to circadian oscillation the analysis with 1D oxyblot was started.

4.3. Determination of Carbonylated Proteins

The aim was to determine which were the most interesting times of the circadian cycle for the analyses in 2D electrophoresis. The principal objective was to determine the maximum and minimum of carbonylated proteins level in the circadian cycle.

4.3.1. Quantification carbonylated proteins in 1D electrophoresis

1D electrophoresis was made to quantify the level of carbonylated proteins during two treatments, the unsynchronized and synchronized cells, as it can be seen in Figure 13. First we can observe the pictures which were taken to the membranes showing the total of protein (red) with a solution Fast green and revealed at 700 nm and the carbonylated proteins (green) revealed at 800 nm in control cells (picture A) and cells synchronized with serum shock (picture B).

The graphics correspond to the level of carbonylated protein in each time of each treatment. The graph in picture A corresponds to two analysis made in the samples subject to a treatment without synchronization. It is possible to see in this graph that the level of carbonylated proteins do not have a rhythmicity. However, in the second graph (in picture B), which corresponds to the combination of 8 batches of synchronized cells, it can be seen that the behaviour of the

carbonylated proteins have a rhythmicity. When these results were analysed making a filting and Fourier transform to see the period, it was discovered that they showed a period of 22,8 ± 7 h, what proves the circadian rhythmycity of the level of carbonylated proteins. It can be observed that the maximum of carbonylated proteins occure at 22 h ± 7 h and 46 h ± 7 h after the beginning of the treatment and minimum are observed at 10 h ± 7 h and 34 h ± 7 h. These points were chosen to detect and quantify the carbonylated proteins through 2D electrophoresis.

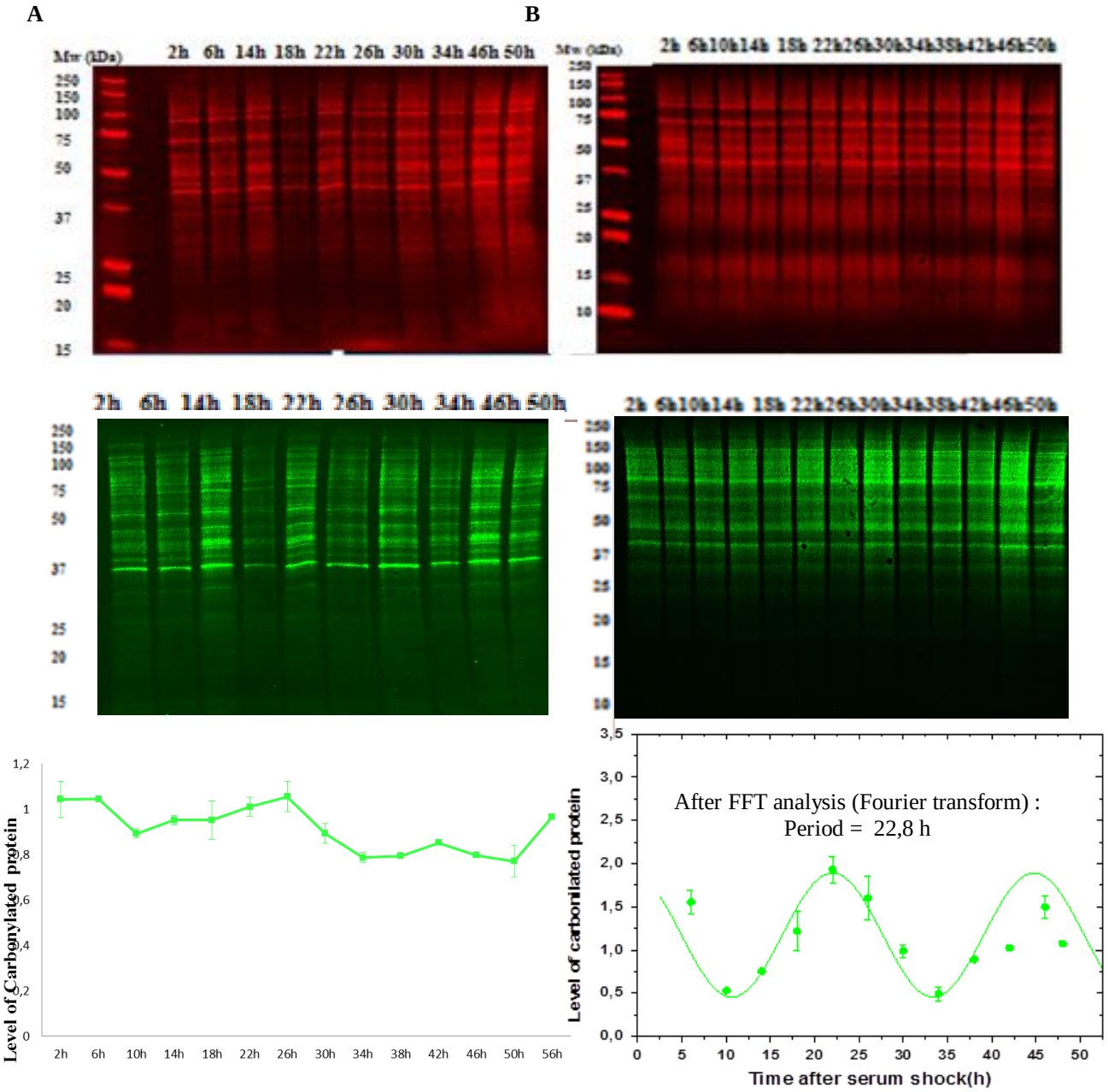

Figure 13- Levels of Carbonylated proteins in HEK-293 cells during the two treatments (without synchronization – WS, corresponding to picture A and synchronization with serum shock –SSS, corresponding to picture B).In red the total of protein using Fast Green solution and in green the carbonylated proteins level. **A**- Level of Carbonylated Protein in cells WS; **B**- Level of Carbonylated Protein in cells SSS analyse with Fourier transform to see the period.

4.3.2. Quantification of Carbonylated Protein in 2D electrophoresis

The 2D electrophoresis is the method of choice to separate proteins because it can simultaneously separate thousands polypeptides of a complex mixture based on two different properties: the electric charge and molecular weight.

Proteomics requires the use of biological samples with a good quality. Indeed, the isoelectric focalisation, the first separation, is highly dependent on the delicate preparation of biological samples which should allow maximum solubilisation of proteins and prevent their aggregation and degradation. That's why in a first step it is necessary to optimize extraction conditions for this type of cells and adjust the conditions before the final analysis of the different times. For this, some HEK-293 unsynchronized were used and cultivated in normal conditions.

4.3.2.1. Analysis of the necessary quantity of protein (ECL method)

First a 2D electrophoresis was made with 13 cm strips (pH gradient between 3 and 11) using a revelation of membrane by Enhanced Chemiluminescence (ECL) method with the aim of verifying the best quantity of protein to be used. This type of method for revelation was chosen because it is the most used and it has a good sensibility allowing us to observe even the small spots. In Figure 14 the results of the three quantities of proteins that were tested can be seen: 150 µg (A), 200 µg (B) and 300 µg (C). When the different gels are compared one can see that even with a lower quantity of protein (A) it is still possible to make quantification of many spots which are very defined. In Figure 15 it can proved that with 150 µg of protein a good Coomassie gel is obtained (Figure 15 A) with well defined spots which will allow to characterise even the small ones.

So, the use of the smallest quantity of protein was chosen because the resolution seemed to be the best one (A). Then with this quantity three different times of exposition (1, 5 and 10 minutes) were also tested and it was observed that the saturation was really quick. Indeed, this method is characterised as using an enzyme which builds to the protein and it sometimes can promote a faster saturation. It is not good to make a quantification of protein because there is a risk of losing some signals and an increase of carbonylated proteins. So this study was continued using the Odyssey revelation that uses fluorescent antibodies because with this one the problems of saturation observed with ECL revelation were avoided. Moreover, the detection of spots by fluorescence has a good sensitivity and linearity of intensity and also a better reproducibility, ease and speed than ECL.

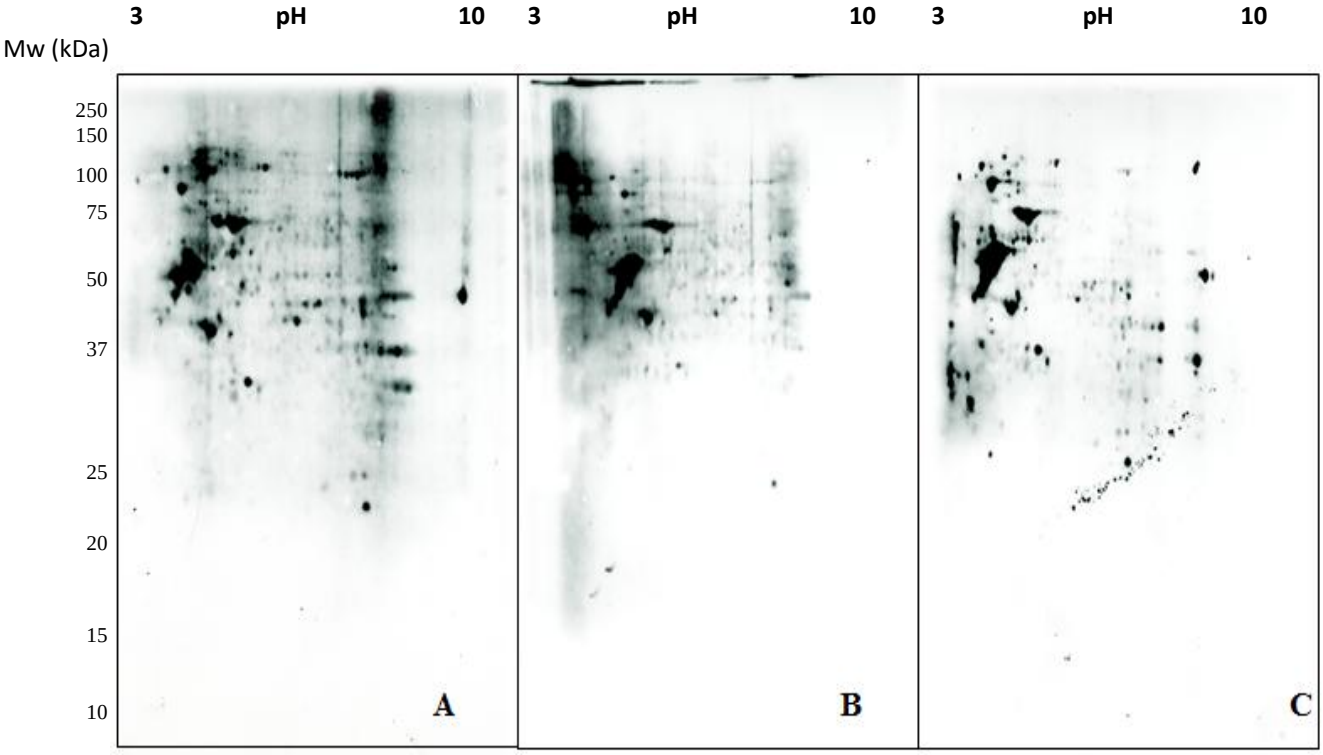

Figure 14- Different quantities of protein tested by Enhanced Chemiluminescence (ECL) method of a sample of normal HEK-293 cells with strips of 13 cm. **A**-150 µg; **B**-200 µg and **C**- 300 µg.

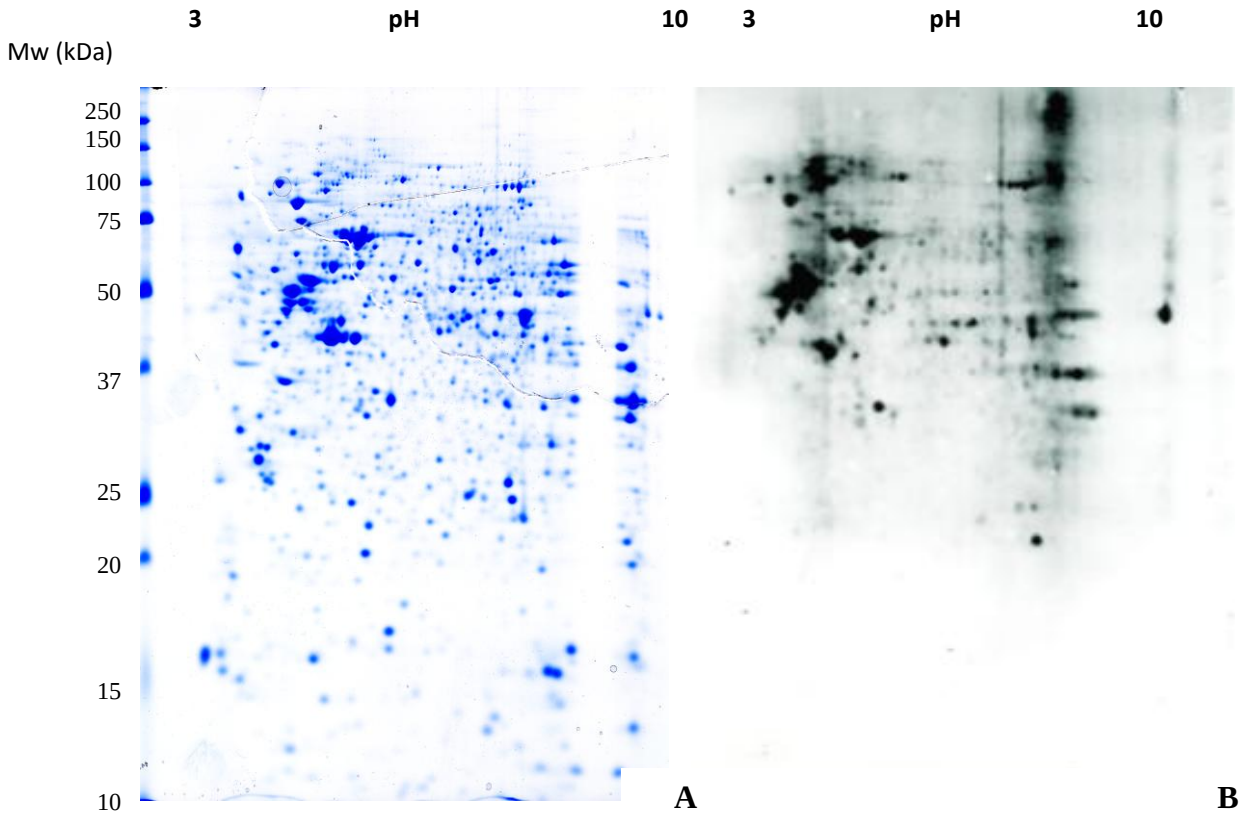

Figure 15- Comparison between Coomassie gel and a nitrocellulose membrane revealed with the ECL method showing the carbonylated proteins of a sample of normal HEK-293 cells using strips of 13 cm. **A**- Coomassie gel (150 µg) **B**- Carbonylated proteins in a nitrocellulose membrane (150 µg).

4.3.2.2. Optimizing of the 2D electrophoresis in HEK-293 cells

An analysis was begun using initially the same amount of protein used with the ECL (150 µg). But the detection with Odyssey is less sensitive which led to the necessity of increasing the quantity of protein to 300 µg (Figure 16 C) to have a more satisfactory signal. Indeed, the pattern of carbonylated with a quantity of 150 µg was very reduced. In the same way there was also a need to increase the concentration of primary and secondary antibodies.

However, when these tests were made a band of salt that worked as a blocking of the separation of the proteins by the isoelectric point was always seen. And it is known that in proteomics it is really important to have biological samples with a good quality. Indeed, some contaminants such as nucleic acids, lipids and salts can disrupt protein separation by two-dimensional electrophoresis. So another Lysis Buffer, (Lysis Buffer II) was tested. In Figure 16 the two types of Lysis Buffers I*[34] (picture A) and II*[35] (picture B) can be compared and it can be observed that with the second one the trace of salt disappears. In this way there is a better focalisation of the proteins, what suggests that the Lysis buffer II is the best to be used. Two types of gels were also tested, a big gel that was usually made (12% of acrylamide) and a small precast gel that was bought from BIO-RAD, Criterion™ TGX Any kD™ Precast Gel. This gel has a non linear gradient of acrylamide concentration which promotes a better separation of proteins from 10-250 kD and a best resolution in 20-100 kD. Besides this, the procedure is faster than with the bigger gel. When the two gels are compared, picture C and D, it can be observed that the biggest gel has more signal of carbonylated proteins than the precast gel. But this gel was made in a different batch of cells so these two global patterns cannot really be compared. However, when the resolution of the spots is observed it can be seen in precast that the spots gel are better defined than in the bigger gel. In pictures E and F the global pattern in the Coomassie gel can be analysed and it can be proved that with the precast gel the spots were very defined but it seems that little problems of focalisation occured, which were subsequently corrected. Concluding, for the 2D electrophoresis the Lysis buffer II was the best for the extraction of proteins and the precast gel can be used for the first verification of variation of carbonylated proteins. However, for the identification of the spots a bigger gel was needed to be made because the spots in precast gel were too small and too close to each other in order to be collected.

[34] (*) the composition is described in attachment 7.3.1.1.
[35] (*) the composition is described in attachments 7.3.1.2.

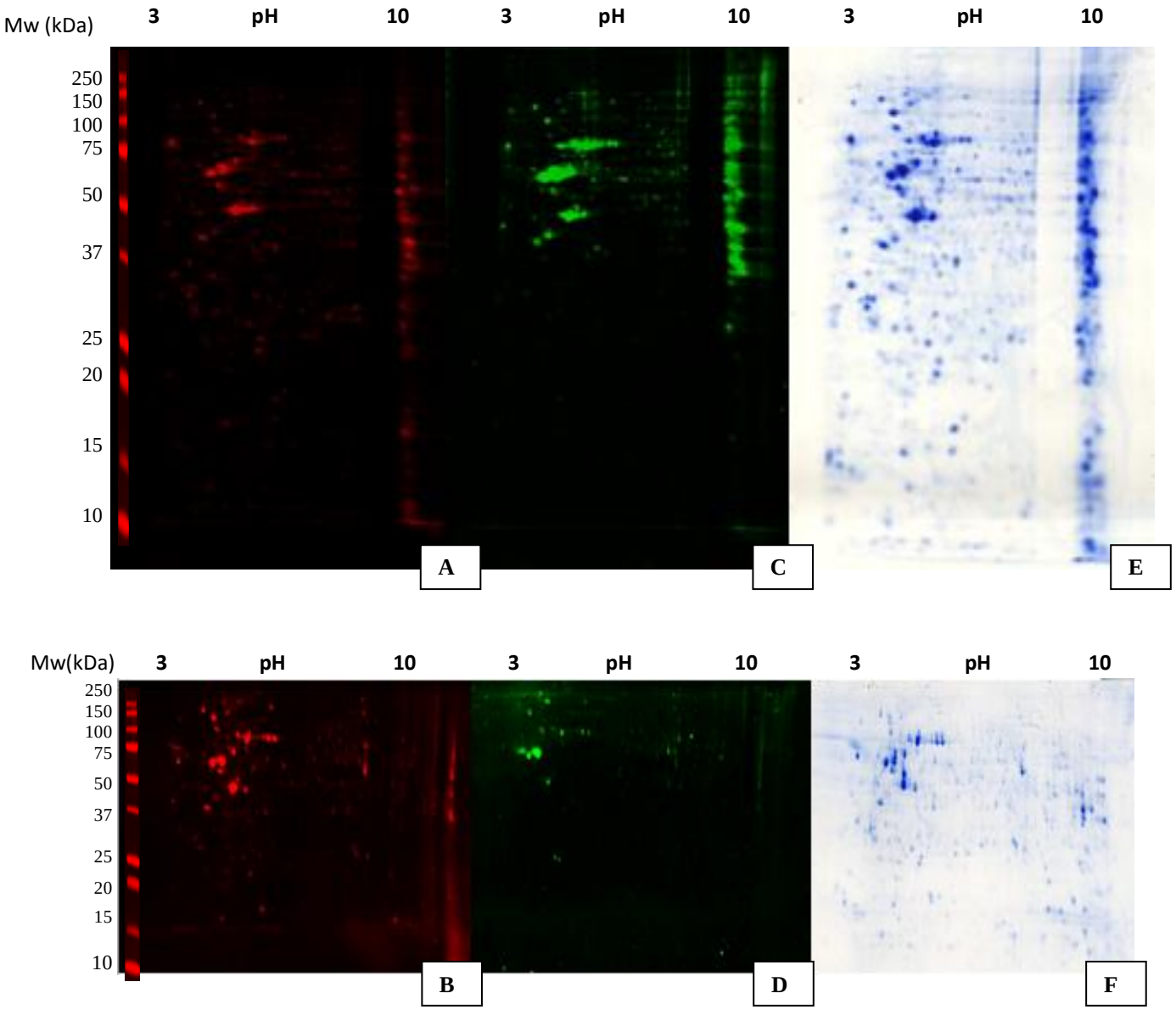

Figure 16- Comparison of different conditions (extraction buffer and sizes of strips) in 2D electrophoresis. **A-** Total of Protein in a sample using Lysis Buffer I and a strip with 13 cm; **B-** Total of Protein in a sample using Lysis Buffer II and a strip with 11cm; **C-** Carbonylated Protein in a sample extracted with Lysis Buffer I and using a strip with 13 cm; **D-** Carbonylated Protein in a sample extracted with Lysis Buffer II and using a strip with 11 cm; **E-** Coomassie gel of a sample extracted with Lysis Buffer I and a strip with 13 cm; **F-** Coomassie gel of a sample extracted with Lysis Buffer II and a strip with 11 cm.

4.3.3. *Level of carbonylated proteins at different times*

To quantify the level of carbonylated proteins in 2D electrophoresis, unsynchronized cells (Figure 17 A, B) and synchronized cells (Figure 17 C, D) were used. The total of protein and the carbonylated proteins of these samples after 2 h of serum shock treatment were compared. After the analysis of the spots with the 2DPlatinium programme (Figure 18), it was observed that the global carbonylation related to the global protein expression of these two samples was very close. Indeed, for the unsynchronized cells there were 15,38% of the carbonylated proteins and for synchronized cells 14,29%. Carbonylated proteins are only considered increasingly modified when the value is higher than 1,3 because it is a minimum level for significant increase which comes from a recent study of the oxidative-stress induced proteome alterations in human myoblasts (Baraibar, *et al*., 2011). Interestingly, as shown in the Figure 18, proteins with higher carbonylation than in the synchronized cells (2 h) were found. Moreover, in unsynchronized cells there are some proteins that are 10 fold more oxidized. But this is not the most interesting circadian time. Indeed, the aim being to look carbonylation peak observed in 1D electrophoresis.

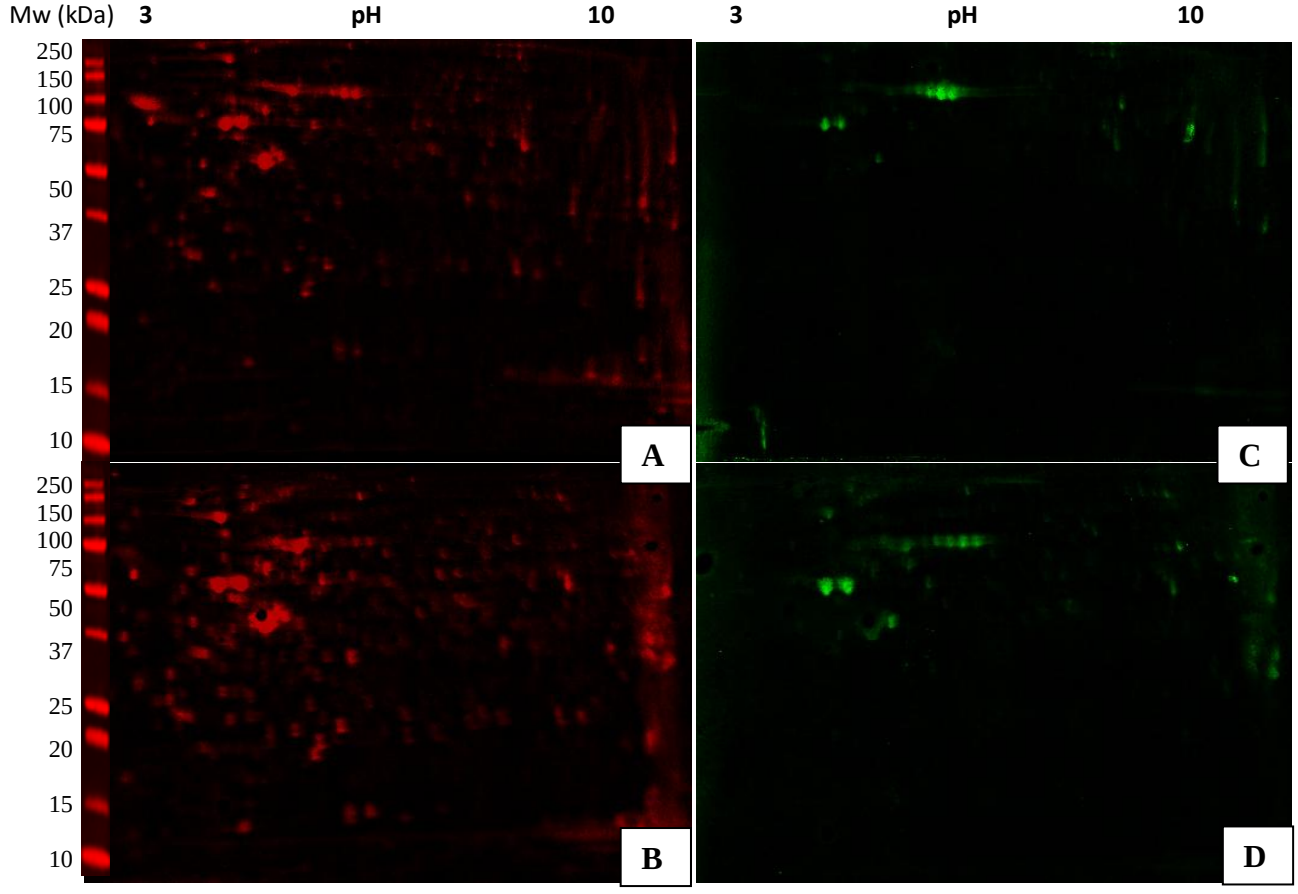

Figure 17- 2D electrophoresis of total of protein and carbonylated proteins in a sample of HEK-293 2 h after the start of the treatment (without synchronization and synchronized by serum shock) **A-** Total of protein in a sample of HEK-293 after 2 h of treatment WS, **B-** Total of protein in a sample of HEK-293 after 2 h of treatment SSS, **C-** Carbonylated protein in a sample of HEK-293 after 2 h of treatment WS and **D-** Carbonylated protein in a sample of HEK-293 after 2 h of treatment SSS.

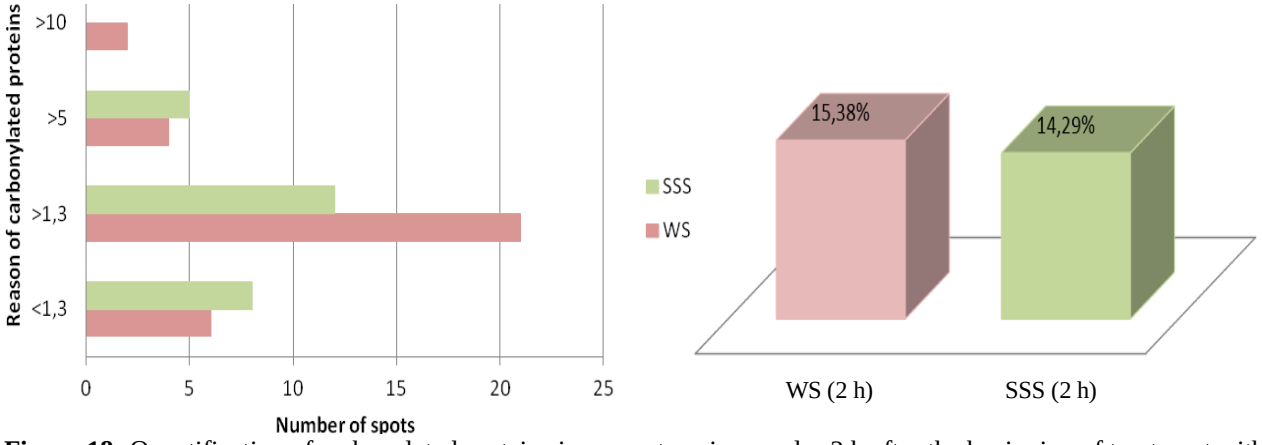

Figure 18- Quantification of carbonylated proteins in percentage in samples 2 h after the beginning of treatment without synchronization (WS) and synchronization with serum shock (SSS) and the number of spots identified as carbonylated proteins in each sample. Percentage of carbonylated spots with oxy ratio bigger than 1,3 related with total protein spots.

To proceed this study the 2D electrophoresis in four times of synchronized cells that correspond to two peak maximum (26 h and 46 h) and two peak minimum (14 h and 34 h) of the carbonylated protein level were tested. After the quantification of the carbonylated protein and the comparison of the total carbonylated proteins between time 14 h (Figure 19 A, B) and the time 26 h (Figure 19 C, D) it was observed that the previous results which were obtained with 1D electrophoresis were correct. Indeed, there are more global carbonylated proteins in time 26 h (12,23%) than in time 14 h (9,59%) (Figure 20). In the same Figure there is a representation of the number of spots identified as carbonylated proteins, calculated with a ratio (carbonylated protein/total protein) bigger than 1,3. Interestingly we can observe that we have some specific proteins that are more carbonylated (time 26 h).

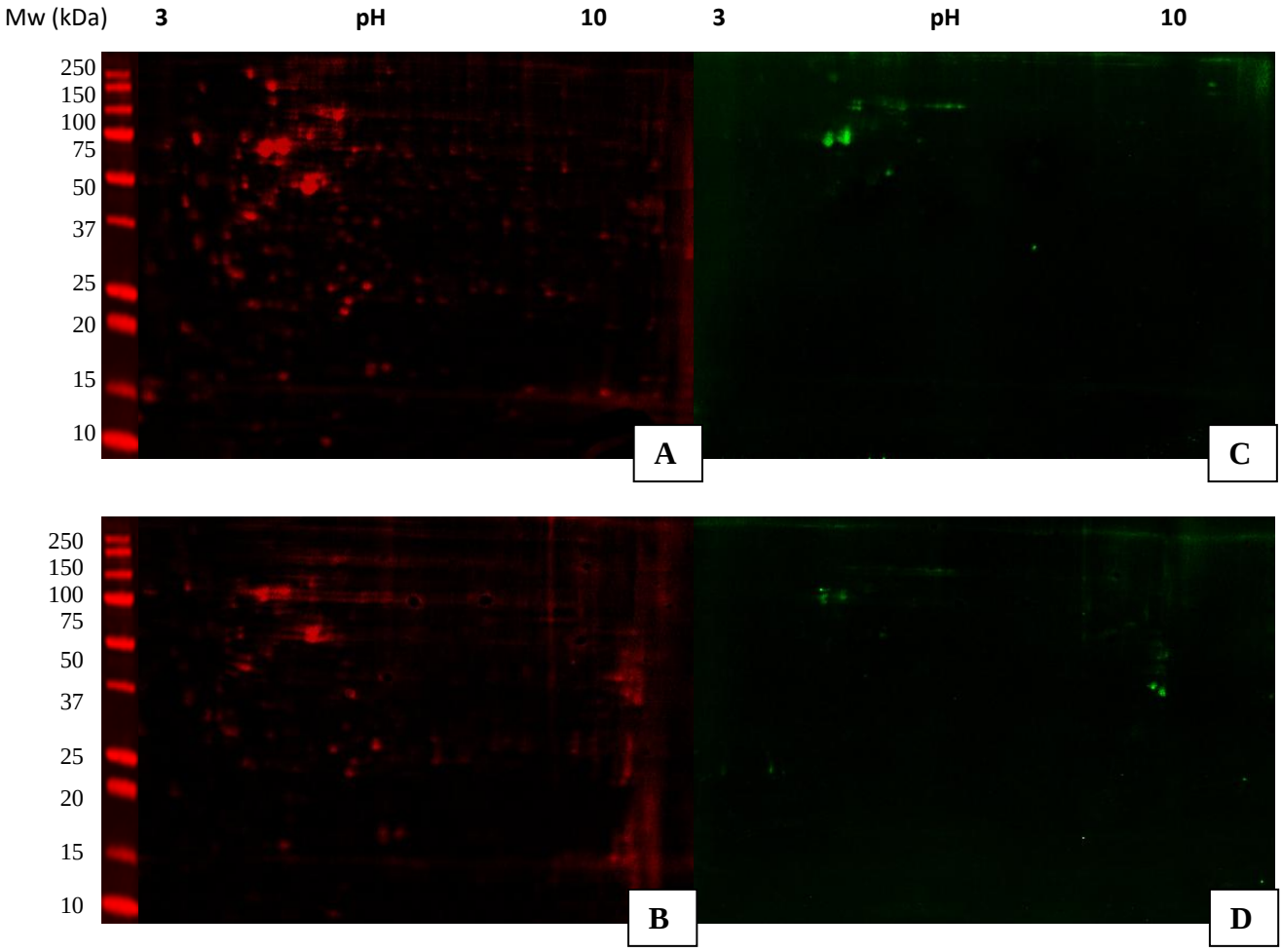

Figure 19-2D electrophoresis of total of protein and carbonylated proteins in a sample of HEK-293 14 h and 26 h after the start of the treatment (synchronization by serum shock). **A-** Total of protein in a sample of HEK-293 14 h after the treatment SSS, **B**- Total Carbonylated protein in a sample of HEK-293 calls 26 h after the treatment SSS, **C**- Total of protein in a sample of HEK-293 cells 14 h after the treatment SSS and **D**- Carbonylated protein in a sample of HEK-293 cells 26 h after the treatment SSS.

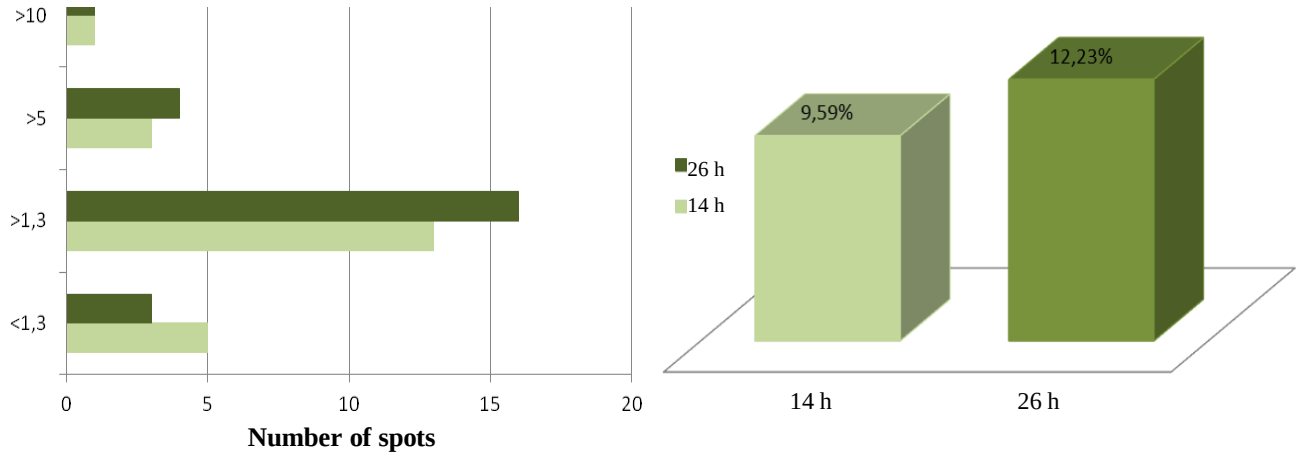

Figure 20- Quantification of carbonylated proteins in percentage in samples 14 h and 26 h after the beginning of treatment synchronization with serum shock (SSS) and the number of spots of the carbonylated proteins. Percentage of carbonylated spots with oxy ratio bigger than 1,3 related with total protein spots.

Next, there was a need to see if there was the same in the second part of the cycle. That is why the times 34 h and 46 h were analysed. In Figure 22 it can be observed that there is a significant increase of the total carbonylated proteins in time 46 h when comparing with time 34 h. Moreover, we have, also, lots of spots in time 46 h with an oxy ratio >1,3. This means that, at this time, there are some particular proteins that have a circadian oxidation.

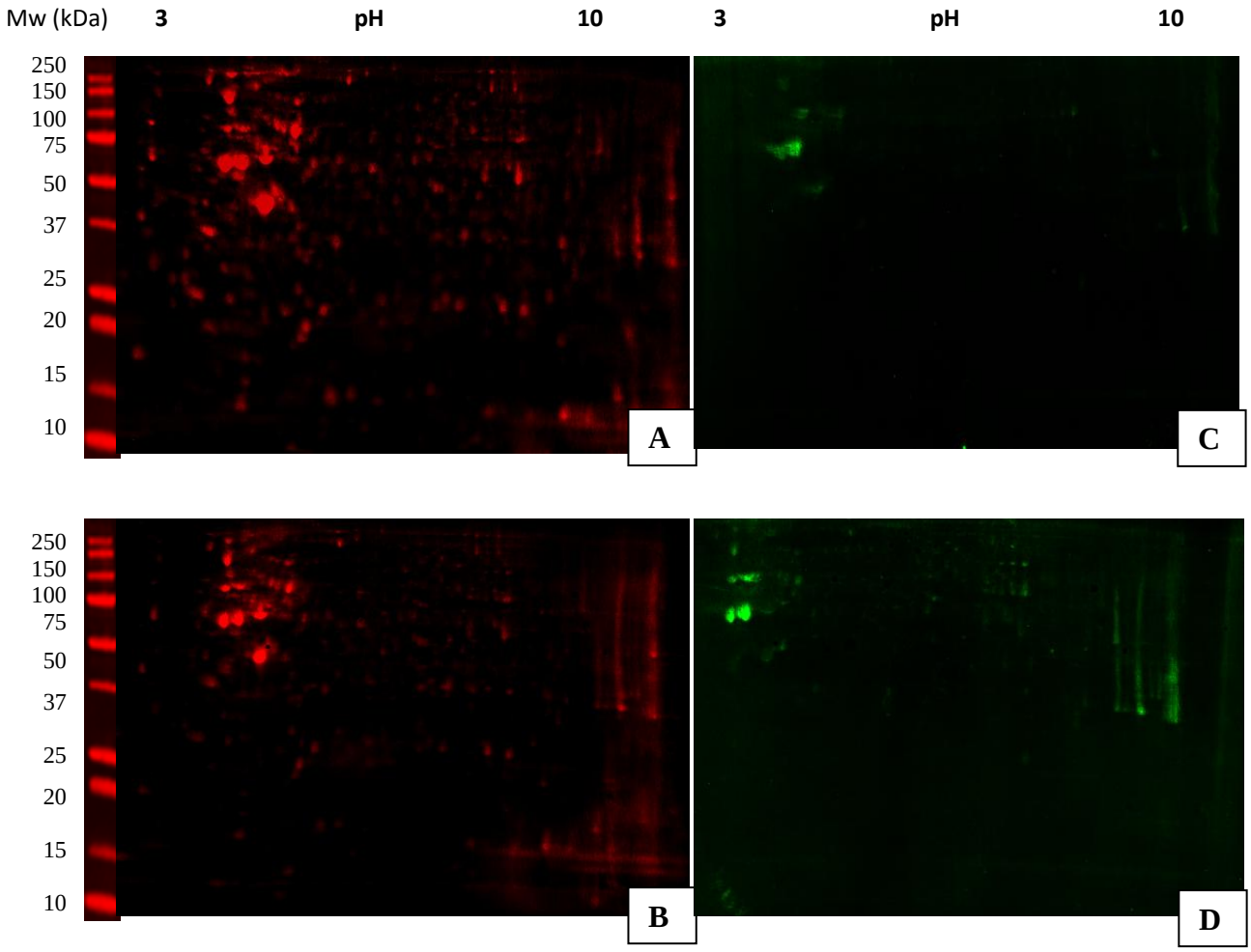

Figure 21-2D electrophoresis of total of protein and carbonylated proteins in a sample of HEK-293 34 h and 46 h after the start of the treatment (synchronization by serum shock). **A-** Total of protein in a sample of HEK-293 34 h after the treatment SSS, **B**- Total Carbonylated protein in a sample of HEK-293 calls 46 h after the treatment SSS, **C**- Total of protein in a sample of HEK-293 cells 34 h after the treatment SSS and **D**- Carbonylated protein in a sample of HEK-293 cells 46 h after the treatment SSS.

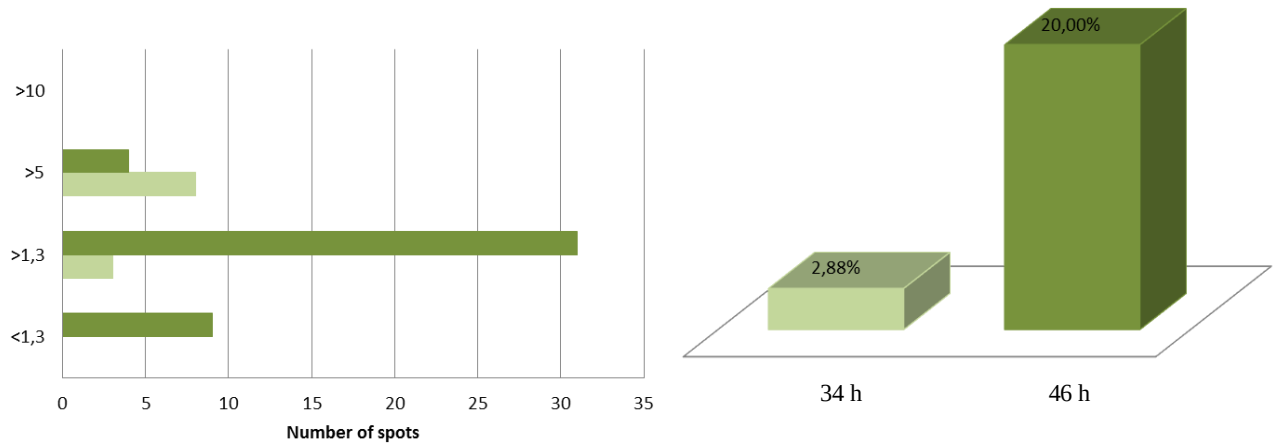

Figure 22- Quantification of carbonylated proteins in percentage in samples 34 h and 46 h after the beginning of treatment synchronization with serum shock (SSS) and the number of spots of carbonylated proteins. Percentage of carbonylated spots with oxy ratio bigger than 1,3 related with total protein spots.

5. DISCUSSION AND CONCLUSION

The circadian rhythm is characterised by being controlled by transcription-translation feedback loop (TTFL) mechanism, which comprises a number of clock genes and their corresponding proteins (CLOCK, BMAL1, PERs, CRYs, REV-ERBα) (Kondratov, 2007; Reppert & Weaver, 2001; Xydous, *et al*., 2012). Due to the development of molecular studies nowadays it is possible to relate a disruption of the circadian cycle with the development of various diseases related to aging (Kondratov, 2007; Lévi, *et al*., 2010; Tevy, *et al*., 2013). A depletion of the proteins BMAL1, PER1 and PER2 causes an increase of the levels of reactive oxygen species (ROS) and, consequently, the occurrence of oxidative stress (Chondrogianni, *et al*., 2012; Dalle-Donne, *et al*., 2003). Moreover, it is known that oxidative stress increases progressively with age and causes changes essentially in proteins (Ahmed, *et al*., 2010; Cecarini, *et al*., 2007; Chondrogianni, *et al*., 2012). Carbonylation is one of the most common injury associated with them and with the identification of these damaged proteins by 2D electrophoresis making it is possible to understand how this modification on proteins could affect cellular function (Ahmed, *et al*., 2010). Results obtained in a previous study performed by Audrey Desvergne reveal that in the culture of cells synchronized by serum shock, the level of carbonylated proteins shows a circadian rhythmicity. This work had the aim of studying the levels of carbonylated proteins along the circadian cycle and finding the principal times of interest to identify the specific oxidized proteins and compare them to those oxidized proteins already associated to cellular aging.

The cells used for this study were HEK-293. These cells were chosen because they are used in many studies, especially protein studies. Besides this, they have lots of advantages: they are easy to maintain and reproduce rapidly allowing to obtain a lot of material for later analysis (He & Soderlund, 2010; Thomas & Smart, 2005). Indeed, the primary studies made by Audrey Desvergne show that serum shock synchronized very well this type of cells. Synchronization with dexamethasone was also tested because it was written in the literature that this glucocorticoid can promote a longer synchronization than serum shock and it is known that it is more relevant to make the analysis after the second cycle of the rhythm. Although Balsalobre and his co-workers claim that in rat fibroblasts the expression of circadian clock genes can resist until 3 days in medium without serum after synchronization, it was observed that this type of synchronisation induces a cellular stress. Taking into account these results it can be said that the synchronization with serum shock can be the preferred for HEK-293 cells because like this there is no risk of making a wrong analysis of the level of carbonylated proteins. When the clock genes transcripts was analysed with a Fourier transform it was observed that HEK-293 cells have a circadian period between 20 h and 27

h. Moreover, as a control, normal cells were submitted to a medium without serum, in order to verify if these conditions alone induced a circadian synchronisation. This serum deprivation is used to inhibit the cell cycle and to confirm that, the period of *Cyclin B1* transcript was analysed and it was seen that the cell cycle has a period of 52 h. Hence it has been evidenced that our findings are directly related to the circadian rhythm and not to the cell cycle.

The 1D electrophoresis followed by oxi-blot was performed to observe the level of carbonylated proteins throughout the different treatments. The quantification was made in cells both unsynchronized (Figure 13 A) and synchronized (Figure 13 B) taking into account the total number of proteins. Comparing the charts it was observed that different samples without synchronization, show a non circadian variation of the values of damaged proteins. In graph B, which corresponds to the level of carbonylated analyse after fitting, oscillations in two well-defined circadian cycles can be observed. It's starting around 10 h after the serum shock treatment. In addition, after analyse by Fourrier transform it could be proved that the period of the level carbonylated proteins is about 22,5.

2D electrophoresis allows the separation of proteins not only by its molecular weight but also by its isoelectric point. It is therefore considered as a very advantageous method to detect and quantify level of carbonylation in each protein. After the optimisation of all the conditions necessary to have a good separation and resolution of spots, the carbonylated protein was quantified and compared between the times selected with the 1D oxyblot. Interestingly, some particular proteins that were more oxidized in the time 26 h and 46 h with an oxy ratio up to 1,3 were found. In addition, it could be observed that during the second cycle (time 46) we have a greater number of spots with a ratio up to 1,3 than in the first cycle.

Taking into account all the results, it can be concluded that: 1) dexamethasone induced a cellular stress, also like the starvation of serum; 2) serum shock did not induce a cellular stress and synchronized this type of cells very well, with a periodicity of the clock genes between 20 and 27 h and a periodicity of the level of carbonylated proteins around 23 h; 3) existed to define circadian cycles with a minimum of the level of carbonylated proteins at 14 and 34 h and a maximum at 26 and 46 h but the better to use for the identification of proteins with a combined use of mass spectrometry and database is the second circadian cycle.

Indeed, as future perspectives it would be good to make the identification of the specific target proteins involved in the circadian oxidation by mass spectrometry so that later there could be a comparison of themes to the biological biomarkers of situation of oxidative stress and cellular aging.

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7. ATTACHMENTS

7.1. Solutions for cell culture

7.1.1. *Culture medium (DMEM-PSG)*

The medium to make a cell culture, usually called by DMEM-PSG, consists in 500 mL of Dulbeco's Modified Eagle Medium (DMEM) supplemented with 1% of *Penicillin-streptomycin* (P), 1% of L-Glutamine (G) and 10% of Fetal Bovine Serum (S or FBS). All of the components to make this specific medium were from SIGMA®-ALDRICH and were stored in a refrigerator at 4°C.

7.1.2. *Medium without serum (DMEM-PG)*

The medium without serum that was used to incubate the cells when a synchronization was made was composed by 500 mL of DMEM with 1% *Penicillin-streptomycin* (P) and 1% of L-Glutamine (G). The reagents were from SIGMA®-ALDRICH and stored in a refrigerator at 4°C.

7.2. Solutions for quantitative PCR

7.2.1. *Lysis Buffer*

The Lysis Buffer used to extract the RNA that were in the samples was composed by 350 µL of RA1 solution and 3,5 µL of β-mercaptoethanol for each sample. The RA1 comes in the Kit that was used to make the extract of RNA (NucleoSpin® RNA II kit by MACHEREY-NAGEL) and β-mercapoethanol comes from SIGMA®-ALDRICH.

7.2.2. *TAE solution*

The solution TAE 50X was made with a dilution of 242 g of Trisbase with 100 mL of EDTA 0,5 M, 57,1 mL of acetique acid and MilliQ water was added until 1 L. When a solution more diluted (TAE 1X) was needed, 20 mL of TAE 50X was added in a bottle with 980 mL of MilliQ water. The reagents that were used come from SIGMA®-ALDRICH.

7.2.3. *DNA Dye 6X*

To prepare DNA Dye 6X solution necessary lots of steps were followed. The first steps consisted in an addition of 25 mg of bromophenol blue in 6,7mL of MilliQ water and solution was mixed and only then 25 mg of xylene cyanol FF was added. Finally 3,3 mL of glycerol was added and the solution was mixed very well and the aliquots were prepared and saved at -20ºC. All the reagents that were used come from GE HEALTHCARE.

7.2.4. *DNAmix*

The solution DNAmix was prepared with a conjugation of various solutions that come in a specific kit called by SuperScript® III First – Strand Synthesis System for RT-PCR (Invitrogen, LIFE TECHNOLOGIES). This mixture was composed by 2 µL of solution Buffer, 4 µL of solution magnesium chloride ($MgCl_2$), 2µL of solution Dithiothreitol (DTT), 1 µL of solution RNase out and 1 µL of solution SuperSIII.

7.2.5. *Standard curve*

Five tubes labelled with 5, 10, 20, 50 and 100, respectively, were placed, corresponding to the number of dilution of each sample. In the first one, 120 µL RNA-free water and 10 µL of 3 different cDNA not diluted (RNA extracted of other samples) were added and homogenized. In the tubes 15; 22,5; 27 and 28 µL of the solution of tube 1 was added (Table.1). Each was homogenized and stored at -20°C until it was used.

Table 2- Summary of the preparation of the different samples, with different concentrations to make a standard curve.

	Sample 1 (Dilution 5x)	**Sample 2 (Dilution 10x)**	**Sample 3 (Dilution 20x)**	**Sample 4 (Dilution 50x)**	**Sample 5 (Dilution 100x)**
Sample 1	-	15 µL	7,5 µL	3 µL	2 µL
cDNA 1	10 µL	-	-	-	-
cDNA 2	10 µL	-	-	-	-
cDNA 3	10 µL	-	-	-	-
RNA-free water	120 µL	15 µL	22,5 µL	27 µL	28 µL

7.2.6. ***Solutions primers***

To examine with qPCR, 8 µL of *primer* solution was needed for each well. To prepare the solution *primer* (8 µL) 1 µL of *primer sens*, 1 µL *primer antisens*, 1 µL RNA-free water and 5 µL SyBr Green I (LightCycler480, SyBr Green I Master, ROCHE) were added and homogenized. It was important to always have the solutions in ice.

7.3. Solutions for 1D and 2D electrophoresis

7.3.1. ***Lysis Buffer***

7.3.1.1. Lysis Buffer I

To make a final volume of 10 mL it was necessary to weigh 4,8 g of Urea, 1,58 g of Thiurea, 400 mg of Chaps 4% and 30 mg of DTT and dissolve everything with Tris 10 mM solution (pH around 7,4). All the reagents to make the Lysis Buffer were bought from SIGMA®ALDRICH with the exception of DTT which was purchased from GE HEALTHCARE.

7.3.1.2. Lysis Buffer II

To prepare a Lysis buffer II, 4,8 g of Urea and 1,58 g of Thiurea were weighed and dissolved in 4 mL of MilliQ water. After 0,1 g of Amberlyte was added and this solution stayed in agitation during 10 minutes. Next the final solution with a dilution 1:10 of the Tris 1 M and the previous solution with 40 mg of Chaps and 3 mg of DTT by 1 mL. All the reagents were bought from GE HEALTHCARE. with the exception of Chaps and Thiurea which were purchased from SIGMA®-ALDRICH.

7.3.2. ***Gel from Oxyblot 1D***

7.3.2.1. First gel

To make just one first gel 5,45 mL of MilliQ water, 3,2 mL of Tris 1,5 M (with pH=6,8), 128 µL of Sodium dodecyl sulfate (SDS) 10%, 3,9 mL Acrylamide, 146,3µL Ammonium peroxodisulfate (APS) 10% and 9,75 µL of Temed was added and mixed. All the components of gel were bought from SIGMA®ALDRICH with the exception of SDS, Acrylamide and Temed which were purchased from BIORAD.

7.3.2.2. Second gel

The second gel consisted in an addition of 2,5 mL of water, 935 µL of Tris 0,5M (pH 6,8), 37,5 µL of SDS 10%, 350 µL of Acrylamide, 75 µL of APS 10% and 10 µL of Temed. Like in the first gel, all the components of gel were bought from SIGMA®ALDRICH with the exception of SDS, Acrylamide and Temed which were purchased from BIORAD.

7.3.3. ***Migration buffer***

For the preparation of 1 L of migration buffer 10X it was necessary to weigh about 30 g of Trisbase, 144 g of Glycine and 100 mL of SDS 10%. The reagents came from SIGMA®-ALDRICH.

For the electrophoresis migration buffer 1X was used, which was prepared with 100 mL of migration buffer 10X and 900 mL of MilliQ water.

7.3.4. ***Transfer buffer***

There was a preparation 1 L of transfer buffer 10X with 30 g of Trisbase and 144 g of Glycine which derived from SIGMA®-ALDRICH. When a transfer started, a transfer buffer dilueted, which was composed by 100 mL of transfer buffer 10X, 200 mL of ethanol absolute and 700 mL of MilliQ water was used.

7.3.5. ***PBS 10X***

The PBS 10X was made with 80 g of sodium chloride (NaCl), 26,8 g of disodium hydrogen phosphate (Na_2HPO_4 $(H_2O)_7$) and 2,4 g of Potassium di-hydrogen phosphate (KH_2PO_4) were weighed. It was completed with water until making 1 L and after the pH was adjusted around 7,4±0,2. All the reagents were purchased from SIGMA®-ALDRICH with the exception of KH_2PO_4 that came from MERCH. When PBS 1X was needed, it was necessary to make a dilution with 100 mL of PBS 10X in 900 mL of MilliQ water.

7.3.6. ***Washing Buffer***

The washer buffer was prepared with the addition of 100 mL of PBS 10X, 1 mL of Tween and completed with MilliQ water until 1 L. The Tween was bought from SIGMA®-ALDRICH.

7.3.7. ***Fast Green Solution***

The Fast Green solution was made with a cocktail of reagents, 60 mL of ethanol absolute, 20 mL of acid acetic, 120 mL of MilliQ water and 0,02 g of fast green. All the components came from SIGMA®-ALDRICH. It was important to pay attention to protect this solution from the light.

7.3.8. ***Blocking Buffer***

7.3.8.1. Odyssey Buffer

The Odyssey Buffer was prepared with a dilution 1:2 of Blocking Buffer (THERMO SCIENCE) and PBS 1X.

7.3.8.2. BSA Buffer

The BSA Buffer was prepared with 1 g of BSA (SIGMA®-ALDRICH) in 100 mL of Washer Buffer.

7.3.9. ***Re-Hydration Buffer***

For 1 mL of Re-hydratation Buffer it was necessary to add 420 mg of Urea, 152 mg of Thiurea, 40 mg of Chaps, 12 µL of Destreak, 5 µL of Pharmlyte™ 3-10 for IEF and just a point cone of Blue Bromophenol. The Urea, Destreak and Blue Bromophenol came from GE HEALTHCARE, the Pharmlyte came from BIOSCIENCES and the rest came from SIGMA® ALDRICH.

7.3.10. ***Derivatization Solution***

The derivatization solution for 2 strips was made with an addition of 40 mg of 2,4-dinitrophenylhydrazine (DNPH) from SIGMA®-ALDRICH, 1,5 mL of hydrochloric acid (HCl) from PRO LAB and MilliQ water was added until completed 20mL.

7.3.11. ***Neutralization Solution***

To prepare the neutralization solution for 2 strips it was necessary to weigh 12,114 g of Trisbase from SIGMA®-ALDRICH and this was dissolved in 35 mL of MilliQ water and 15 mL of glycerol from GE HEALTHCARE.

7.3.12. ***Equilibration Solution***

The equilibration solution for 2 strips (50 mL) was made with a dilution of 18 g of Urea in 5 mL of solution Tris 0,5 M (pH 8,8) and 10 mL of MilliQ water. After 5 mL of SDS 10% and 15 mL of Glycerol were added.

7.3.12.1. Solution B1

The solution B1 was made with 20 mL of equilibration buffer with 0,2 g of DTT (SIGMA® ALDRICH).

7.3.12.2. B2 Solution

The B2 solution was made with 20 mL of equilibration buffer with 0,6 g of iodoacetamide from SIGMA®-ALDRICH and a little of Blue Bromophenol from GE HEALTHCARE.

7.3.13. *Gels for Oxyblot 2D*

The 13 cm strips used in this work needed a gel made by us, however the gels for the smaller strips (11 cm) were precast gels (Criterion™ TGX Any kD™ Precast Gel) bought to BIO RAD. To make two gels it was necessary to have 52 mL of MilliQ water, 36 mL of Acrylamide 40%, 30 mL of solution Tris 1,5 M (pH around 8,6), 1,2 mL SDS 10%, 0,6 mL of APS 10% and 60 µL of Temed. Then a little quantity of isopropanol was placed to have a good front of migration.

7.3.14. *Fixation Buffer*

To prepare 1,5 L of fixation buffer 450 mL of ethanol absolute and 30 mL of phosphoric acid (H_3PO_4) from SIGMA®-ALDRICH were added.

7.3.15. *Pre Coloration Buffer*

To make 1 L of pre coloration buffer it was necessary to have 120 g of ammonium sulfate ($(NH_4)_2SO_4$), 20 mL of phosphoric acid and 20 mL of ethanol absolute. All the reagents came from SIGMA®-ALDRICH.

7.3.16. *Coloration Buffer*

The coloration buffer was made with 250 mL of pre-coloration buffer and 1,25 mL of Coomassie Blue Solution. To make the Coomassie solution 2 g of Blue Coomassie G250 (GE HEALTHCARE) were weighed and 100 mL of previously heated MilliQ water was added. This was dissolved at 100ºC with agitation and after cooling azide sodium solution (0,2 g/L) was added.