EVALUATION OF THE IMPACT OF GRAPE SEED PROANTHOCYANIDINS EXTRACT (GSPE) CONSUMPTION ON BIOMETRIC PARAMETERS IN AN ANIMAL MODEL OF CHRONODISRUPTION

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ABSTRACT

Background: Biological processes are regulated internally by the **central** and **peripheral clocks** following cycles of 24 hours (**circadian rhythms**). This synchronization is mostly governed by the external signals of light and darkness that set the internal clocks through the nervous system. It has been seen that the alteration of these circadian rhythms (**chronodisruption**) is closely related to serious **cardiometabolic diseases**. **Phenolic compounds,** including grape seed proanthocyanidins extract **(GSPE),** have shown to be important modulators of the biological rhythms**.**

Objectives: To study the changes induced in a chronodisrupted animal model and evaluate the ability of GPSE to alleviate the alterations generated.

Results: A decrease in body weight and food intake, a lipidic plasma profile altered with a significant increase in TAG, VLDL, phospholipids and glycerol were observed in the disrupted animals.

Conclusions: The animal model was useful to identify chronodisruption biomarkers, but the administered dose of GSPE could not palliate the circadian disruption.

ABBREVIATIONS

AC: Atherogenic coefficient

AI: Adiposity index

AIP: Atherogenic index

AMPK: AMPK-activated protein kinase

ATGL: Adipocyte TG lipase

BAT: Brown adipose tissue

BMAL1: Brain and Muscle ARNT-like protein 1

BMI: Body Mass Index

CAT: Catalase

CCG: Clock controlled genes

CCK: Cholecystokinin

CLOCK: Circadian locomotor output cycles kaput

COMT: Catechol-O-methyltransferases

CVD: Cardiovascular disease

DIO: Diet-Induce obesity rats

ECGC: Epigallocatechin gallate

EWAT: Epididymal adipose tissue

FFAs: Free fatty acids

GLP-1: Glucagon-like peptide 1

GSH: Glutathione reduced

GSH-Px: Glutathione peroxidase

GSPE: Grape seed proantocyanidin extract

GSSG: Glutathione oxidized

GST: Glutathione S-transferase

HAT: Histone acetyltransferase

HDAT: Histone deacetylase

HDL: High density lipoproteins

HFD: High Fat Diet

HSL: Hormone-sensitive lipase

IWAT: Inguinal adipose tissue

L11-GSPE: Animals subjected to 11h/11h light/dark cycles treated with GSPE

L11-VH: Animals subjected to 11h/11h light/dark cycle treated with vehicle

L12: Animals subjected to a 12h -12h light/dark cycle

LDL: Low density lipoproteins

LOOH: Lipid hydroperoxides

MDA: Malonaldialdehyde

MetS: Metabolic Syndrome

MWAT: Mesenteric adipose tissue

NAD+: Nicotinamide adenine dinucleotide

NAFLD: Non-alcoholic fatty liver disease

NEFAs: Non-esterified fatty acids

NHc: Lipid fraction without considering HDLc

PYY: Peptide YY

RAAS: Renin-angiotensin-aldosterone system

ROS: Reactive oxygen species

SCN: Suprachiasmatic nucleus

SHR: Spontaneously hypertensive rats

SOD: Superoxide dismutase

SULs: Sulfotransferases

T2DM: Type 2 diabetes mellitus

TAG: Triacylgycerides

TBARS: Thiobarbituric acid reactive substances

TC: Total Cholesterol

TGH: Triglyceride hydrolase

TNFα: Tumor Necrosis Factor-α

TSH: Thyroid stimulating hormone

UGT: Uridine-5'-5-diphosphate glucuronosyltransferases

VLDL: Very low-density proteins

WAT: White adipose tissue

1. INTRODUCTION

1.1. Biological rhythms

Biological rhythms are essential for the maintenance of vital functions in the organisms, participating either directly or indirectly in many key processes such as hormones release, eating habits, digestion, and regulation of body temperature. These rhythms, which include circadian (24-hour) and circannual (12-month) cycles, allow the organisms to optimize their behaviour (Arola-Arnal et al., 2019).

For instance, during the day, the organisms need high levels of energy while at night this requirement decreases. Therefore, it is necessary the existence of an internal mechanism to regulate these needs. In this regard, cortisol levels in the morning are higher than in the evening because its function is to prepare the organisms for their energetic requirements during the day by activating different catabolic pathways with the objective of obtaining energy resources. Inversely, melatonin levels are higher in the night and as the day goes on their levels decrease to get sleep and allocate energy resources to be stored. In addition, the rhythms are necessary to temporarily divide the physiological processes that are incompatible at the same time, such as the simultaneous realization of anabolism and catabolism in the same metabolic pathway.

All these biological rhythms are internally regulated by a molecular clock which, for the aforementioned reasons, it is very important that allow the adaptation of the organisms throughout the day. In conception, these rhythms resulted from the adaptation to different factors, both abiotic (temperature cycles) and biotic (food availability), representing a great evolutionary advantage. Actually, the most primitive organisms, such as cyanobacteria, already presented these regulatory processes, which have evolved until they became what we know today (Arola-Arnal et al., 2019; Potter et al., 2016).

The molecular clock consists of a central clock and a set of peripheral clocks. The first is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and synchronizes the peripheral clocks that are distributed in all the cells of the organism, including key body tissues such as the heart, liver, muscle, and adipose tissue. Through various external signals called “zeitgebers” (translated

literally from the German this word means “time-giver”) such as light, diet, mealtimes, physical activity, or social interaction captured by the SCN, the central clock determines our daily rhythms and metabolism by controlling the body functions thanks to the synchronization of peripheral clocks (Zimmet et al., 2019). These external inputs regulate the rhythmic transcription of clock genes which are responsible for controlling the circadian activity of all the organisms. Hence, the regulation of clock proteins at a post-transcriptional level confers another stage of tissue-specific metabolic control, allowing the homeostatic regulation of glucose, lipogenesis, oxidative metabolism, sterol turnover and respiration, among other processes (Bass & Takahashi, 2010).

Light is captured by the organism through a multisynaptic pathway that goes from the SCN to the pineal gland, a gland through which photoperiodic information is disseminated (**Figure 1.**). During the dark phase, the pineal gland synthesizes melatonin, a hormone that increases sleep propensity and acts on its widely expressed receptors to provide photoperiodic information and contribute to the synchronization of circadian rhythms in other tissues. In addition, there is an endogenous regulation by the SCN itself, which produces its own humoral secretions to support the synchronization of the clocks in other tissues (Potter et al., 2016). These additional SCN secretions also contribute to the rhythmic release of hormones such as glucocorticoids by other tissues, key for many peripheral clocks as they contribute to the restoration of 60% of rhythmic gene transcripts in the liver of mice (Reddy et al., 2007). However, the temperature is also an important regulatory factor since molecular clocks can be swept away by its fluctuations through the thermal shock it produces (Potter et al., 2016).

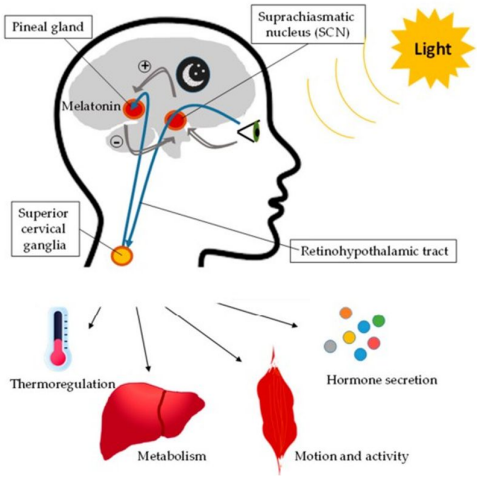


Figure 1. Light acts as the main “Zeitgeber” of the master clock in the hypotalamus. (Arola-Arnal et al., 2019).

1.2. Molecular basis of circadian rhythms

As mentioned above, the circadian system consists of a complex network of molecular clocks located in different body tissues. A hierarchy is established between these clocks in which the central clock is the master regulator primarily responsible to orchestrate the peripheral clocks for maintaining phase coherence within the body's complex network of oscillators through autonomous, behavioural, and humoral mechanisms.

The most accepted molecular mechanism is that the SCN cells contain molecular clocks based on interlocking transcriptional-translational negative feedback loops that generate rhythms of approximately 24 hours in the transcription of the clock genes, which are genes that encode for proteins involved in the regulation of circadian rhythms. These genes temporarily secrete transcription factors that regulate different cellular processes. On the one hand, we find the "Circadian locomotor output cycles kaput" (CLOCK) and, on the other hand, the "Brain and Muscle ARNT-like protein 1" (BMAL1). CLOCK is a transcription factor with a helix-loop-helix motif able to dimerize with BMAL1, being this heterodimer the common central clock in all cells. This stimulates the transcriptional activity of three-period genes (*Per1*, *Per2*, and *Per3*) and two cryptochrome genes (*Cry1* and *Cry2*). The PER/CRY heterodimer acts as a negative feedback loop for the transcriptional expression of CLOCK/BMAL1. In addition, the CLOCK/BMAL1 heterodimer activates the gene expression of *Bmal1*, meaning that the presence of dimerized BMAL1 stimulates its own transcription (Arola-Arnal et al., 2019; Rijo-Ferreira & Takahashi, 2019).

The CLOCK/BMAL1 heterodimer also regulates the expression of the *Nr1d1/2* genes that encode for the REV-ERBa/b nuclear receptors respectively. These nuclear receptors rhythmically repress the transcription of *Bmal1* and *Nfil3*, two genes that are activated by retinoic acid related to the orphan receptor-a/b (RORa/b). REV-ERBa/b and RORa/b act as BMAL1 activators and inhibitors, respectively (Johnston et al., 2016). In addition, PPARa and PGC1a also modulate *Bmal1* transcription through this feedback loop, indicating that REV-ERBa is a nodal point for metabolic input into the clock (Bass & Takahashi, 2010).

Finally, the CLOCK-BMAL1 loop induces the transcription of clock-controlled genes (CCG) that are specific for each cell type. The activity of this loop determines the circadian rhythm of the expression of these CCGs and, therefore, the metabolic and functional cellular rhythms (Arola-Arnal et al., 2019).

In summary, these three mentioned intertwined transcriptional loops regulate most cyclic genes, leading to rhythms in different physiological systems such as sleep or metabolism among others as **Figure 2.** shows.

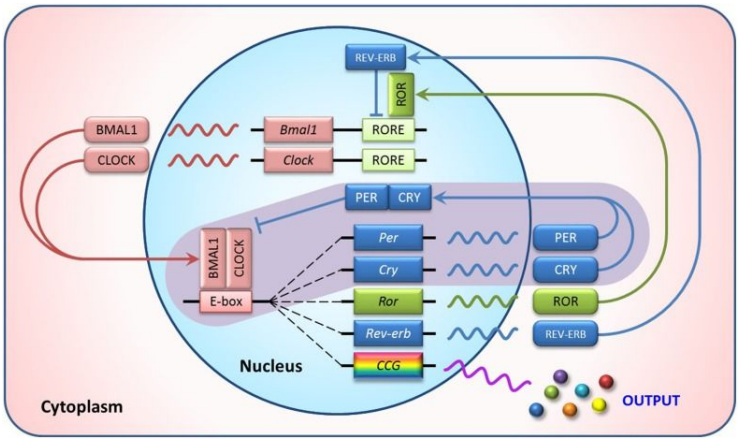


Figure 2. Molecular basis for the regulation of circadian rhythms. (L. Chen & Yang, 2015).

In addition, the transcriptional activator CLOCK also can acetylate histones, in other words, it functions as a histone acetyltransferase (HAT). In this regard, it is involved in the chromatin remodelling in the regulation of gene expression of circadian genes (Doi et al., 2006). In this sense, the compaction of histones certainly prevents the transcription of the genes involved in the maintenance of the circadian rhythm. Since this discovery, it was thought that there must be some component with histone deacetylase (HDAT) activity that counteracts the CLOCK-related HAT activity. Some studies revealed that not all deacetylases succeeded in counteracting the activity of CLOCK until it was observed that SIRT1 could do it (Asher et al., 2008; Nakahata et al., 2008). These results confirmed that, there was an additional loop involving these proteins that use the

stored energy in nicotinamide adenine dinucleotide (NAD+) to catalyse the removal of the acetyl group from substrates as **Figure 3.** shows (Belden & Dunlap, 2008).

It is necessary to highlight that the ratio NAD+/NADH is a direct measure of the energy status of a cell, promoting that the levels of NAD+ oscillate circadianly and so the activity of SIRT1.

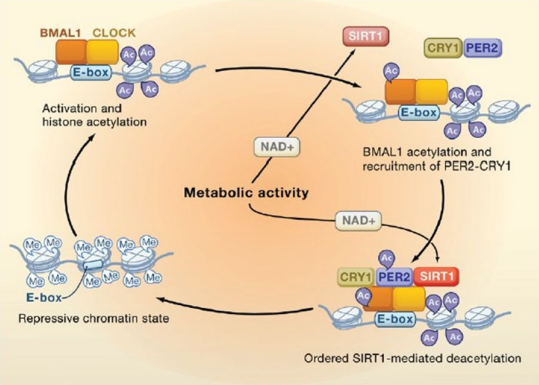


Figure 3. Role of CLOCK activity as a HAT. (Belden & Dunlap, 2008).

In addition to this regulation, AMPK-activated protein kinase (AMPK), which is an enzyme complex that is activated by an increase in the AMP/ATP ratio and serve as a direct measure of the energy status of the cell, also interacts in a circadian-dependent manner with the regulation of the rhythms. AMPK phosphorylates several proteins that participate in metabolic patterns such as CRY1 and casein kinase 1 (CK1) promoting its activation, the phosphorylation of PER and, in turn, its degradation. Also, PER and CRY are phosphorylated by E3 ubiquitin ligase and then directly degraded by the proteasome. **Figure 4.** shows a summary of all the molecular regulations described of the biological clock.

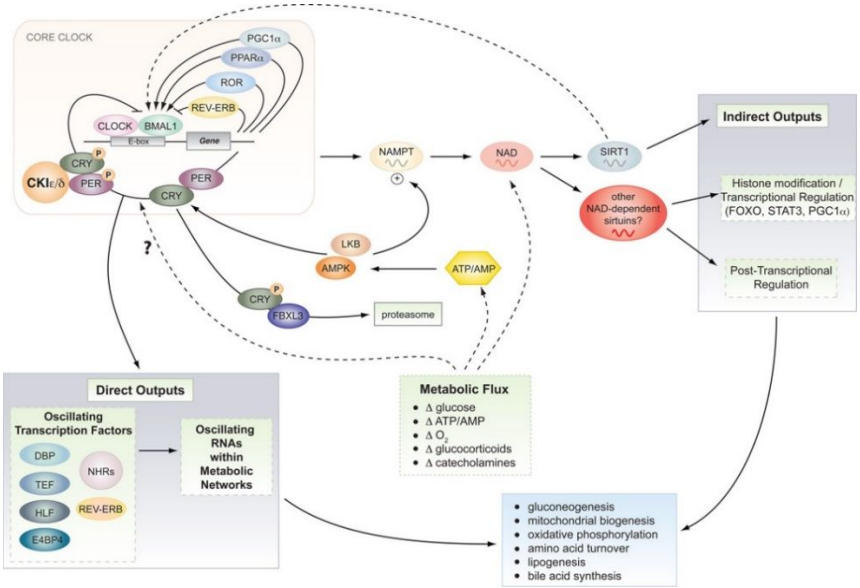


Figure 4. Summary of all the transcriptional and post-translational modifications that syncronize our internal rhythms. (Bass & Takahashi, 2010).

1.3. Chrononutrition: the effect of bioactive compounds

Chrononutrition is an emerging discipline that refers to the intimate relationship between endogenous biological rhythms, nutrition and metabolism, paying special attention to how the time, the composition and size of the meal of each food intake may affect our internal clock system (Arola-Arnal et al., 2019). For example, the time of the meals is highly important for our health, and it has been seen that an altered meal timing can affect several parameters leading to the appearance of several diseases as it is described in more detail below. Moreover, the fact that we have access to products that are not common in our territory or that are out-of-season can alter our rhythms (Arola-Arnal et al., 2019).

Chrononutrition, as a discipline, was first mentioned in 2005 in a Japanese book about nutrition and health (Oda, H et al., 2005), based on the increasing awareness of the impact of the biological clocks on nutrition and, consequently, in health and diseases. In this regard, there are many clinical studies that are focused on the effects of meal timing. Firstly, different common habits linked to the modern lifestyle such as consuming high-energy meals in the evening, skipping breakfast and eat frequently unhealthy snacks or nocturn eating, are associated with a high risk of being overweight or obese, plus other adverse metabolic effects in humans (Aparicio et al., 2017; Hariri & Thibault, 2011). In addition, it is demonstrated that changes in circadian eating patterns causes an increase of energy intake in both humans and rodents (Jakubowicz et al., 2013; Yoshida et al., 2012), reflecting the big problem about obesity and its comorbidities. All these situations are translated into a biochemical background with increased levels of glucose and insulin, as well as increased arterial pressure, decreased leptin levels, leading to an increase of appetite, a reduction of energy expenditure and the alteration of the cortisol secretion rhythm. Secondly, it has been seen that many food components have a high impact modulating the biological rhythms through regulating the expression of clock genes. Among these, phenolic compounds have been seen to counteract this health-related problems (Ávila-Román et al., 2021). Due to the different existing evidence, scientists are making efforts into searching new therapies based on chrononutrition.

1.3.1. Phenolic compounds

As mentioned above, phenolic compounds can interact with biological rhythms. These extensive family of compounds, characterized by its structural heterogenicity, is widely distributed in the plant kingdom. Hence, phenolic compounds can be found in fruits, nuts, seeds, leaves, and roots and in plant-derived products such as beverages (tea or wine), olive oil, and algal-derived products. These compounds are produced by plants when they are under abiotic stress as for example high/low temperatures, drought, UV radiations, the presence of high concentrations of salinity or heavy metals in the soil, among others. In addition, they play an important physiological role in plants, for example, they attract pollinators (Ávila-Román et al., 2021). These molecules are characterized by having at least one hydroxyl group attached to an aromatic ring in their chemical structure. More than 50,000 different compounds have been described and they are classified into two large families: (i) flavonoids, which are subdivided in isoflavones, flavones, flavonols, flavanols, flavanones and anthocyanidins; and (ii) non-flavonoids, including phenolic alcohols, phenolic acids, stilbenes and lignans (Manach et al., 2004).

It is well established that these molecules, especially the metabolites generated by phenolic compounds metabolism, have a lot of benefits for the organisms in terms of health-being. For example, it has been described their anti-inflammatory, antioxidant, anticancer, antibacterial, and anti-Alzheimer's properties, among others (Luca et al., 2020). These activities observed in different scientific studies, point to the potential role of phenolic compounds in the treatment and prevention of different chronic diseases such as diabetes, cardiovascular and neurodegenerative diseases (Ávila-Román et al., 2021). In this regard, a clinical study done with patients with metabolic syndrome (MetS) showed that resveratrol, a stilbene from grapes and wine, significantly decreased weight, body mass index (BMI), fat mass, waist circumference, area under curve of insulin, and total insulin secretion (Méndez-Del Villar et al., 2014). Epigallocatechin gallate (EGCG), representative polyphenol from green tea, has also shown great potential in terms of antioxidant activity and is being evaluated for the management of non-alcoholic fatty liver disease (NAFLD) (Tang et al., 2021). In addition, several studies have shown that curcumin improves the different events

involved in the development of diabetes mellitus type 2 (T2DM) due to its action in the regulation of lipid metabolism (Pivari et al., 2019).

It has been recently seen that the effect of phenolic compounds depends on the time when they are administrated (Torres-Fuentes et al., 2022). For example, the acute administration of resveratrol in male Wistar rats have demonstrated opposite effects in tissue lipoperoxidation depending on the moment when it was administrated, being pro-oxidant when it was administrated during the light period and antioxidant when it was administrated during the night (Gadacha et al., 2009). In C57BL/6J mice, EGCG ameliorated metabolic alterations related to obesity trough the rhythmic regulation of clock genes as *Clock*, *Bmal1* and *Cry* due to the regulation of SIRT1 and PGC1a levels both in liver and in white adipose tissue (Mi et al., 2017). The same time-depending effects are shown in several human studies. One of these studies, showed that in young men the intake of green tea during the night, which is rich in catechins, reduced glucose plasmatic postprandial concentrations but this effect was not shown if the intake was done during the morning (Takahashi et al., 2019). Another study showed that a single dose of a rich polyphenol extract from the algae *Fucus vesiculosus* could reduce the glucose postprandial blood in the night in health woman (Murray et al., 2019).

These differential effects exerted by phenolic compounds are believed to be due to their ability to modulate the circadian rhythms of some factors involved in the onset of cardiometabolic and neurodegenerative diseases such as inflammation, high blood pressure, hyperlipidaemia, or endothelial dysfunction (Torres-Fuentes et al., 2022). Actually, all these factors in turn, present specific circadian rhythms and, when the disease occurs, these rhythms are altered. However, it has been shown that the appearance of these diseases is time-dependent. For example, it has been observed that there is an increase in the frequency of these cardiovascular disease-related hospitalizations and mortality in winter (Karoly et al., 2021).

Despite the great potential of phenolic compounds, their bioavailability is quite low and is affected by different parameters such as external and internal individual factors including sex, age, health status, dose, length of the treatment, etc. (D'Archivio et al., 2010; Margalef, Pons, et al., 2017; Margalef, Pons, et al., 2017). In this regard, there is an important interest in the study of phenolic

compounds for both food and nutraceutical industry sectors to provide new products. For example, it was published a study that supports the use of phenolic compounds from olive oil as nutraceutical addressed to prevent and manage cancer and cardiovascular diseases (CVD) (Reboredo-Rodríguez et al., 2018).

1.3.2. Grape seed proanthocyanidins extract

The polyphenol family of flavanols include two different groups depending on its structure complexity. On the one hand, monomers such as catechin and epicatechin are abundant in different types of fruit as apricot as well as in wine, green tea and chocolate. On the other hand, proanthocyanidins, also known as condensed tannins, are polymers of monomers bounded between C4 and C8 (or C6). When these compounds are mixed with salivary proteins are responsible of the astringent character of wine, cider, tea or beer, and the bitterness of chocolate (Manach et al., 2004).

Grape seed proanthocyanidins extract (GSPE) is obtained from the seeds of grapes (**Figure 5.**) and, how its name says, is rich in proanthocyanidins. This extract is promoted as a dietary supplement for various conditions, including venous insufficiency, promoting wound healing, and reducing inflammation (Grape Seed Extract, NCCIH). The research on this type of phenolic extract it is still growing and offering new insights for using GSPE for treatment and management of several diseases.

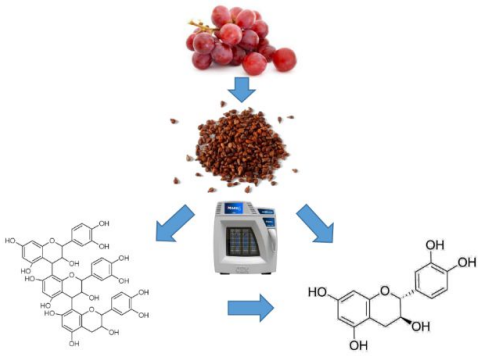


Figure 5. Grape Seed proanthocyanidins extract is obtained from grape seed following different procedures. It includes both polymeric forms and monomers. (J. Chen et al., 2020).

In this regard, several epidemiologic studies have linked the consumption of proanthocyanidins with a lower incidence in some health problems as hypertension and CVD. One of the first studies on this topic by French epidemiologists in 1980. They observed that, despite of the dietary lifestyle that predominated in France, which is characterized by a high fat intake (cholesterol and saturated fats), the mortality rate was very slow compared to the United

States. This phenomenon was called the French Paradox and was attributed to the French tradition of ingesting red wine in meals, which is characterized by having high concentrations of proanthocyanidins (J. L. Richard et al., 1981; Powell et al., 2010). Another important research was carried out by Hollenberg *et al*. They studied the reason why indigenous people from Kuna, an island located in Panama, had low blood pressure levels even at advanced ages. They saw that this fact was due to the consumption of a cocoa beverage rich in proanthocyanidins because, rather than a genetic explanation. Actually, they observed that the people who went to live to other regions and did not consume this beverage, developed hypertension (Bladé et al., 2016; Hollenberg et al., 1997., 2009).

Nowadays, GSPE's properties are quite well characterized, including antioxidant, anti-inflammatory, anti-amyloid b accumulation, antiproliferative, cardioprotective, hypoglycaemic, antibacterial, and anti-apoptotic properties. In addition, it has been described their capacity of modulating lipid metabolism and the gut microbiota too (Rodríguez-Pérez et al., 2019).

First, related to the antioxidant activity of GSPE, in a study carried out in a mouse model of exhaustive exercise-induced fatigue treated with GSPE for 28 days an increased activity of superoxide dismutase (SOD) and catalase (CAT) was observed in both plasma and skeletal muscle. These two are key antioxidant enzymes, SOD converts oxygen peroxides into water while CAT is important in decomposition of hydrogen peroxide to water and oxygen. In addition, diminished levels of malondialdehyde (MDA) in plasma and in skeletal muscle, which is a marker of lipid oxidative stress was also observed. These results points to a protecting role of GSPE in terms of antioxidants defences (Xianchu et al., 2018). Similar results were observed in obese Zucker rats treated with GSPE for 10 weeks, in which increased antioxidant capacity of the cell was explained by the improvement in the glutathione reduced (GSH)/ glutathione oxidized (GSSG) ratio. In this regard, glutathione is the principal antioxidant of the cells protecting them from reactive oxygen species (ROS). Also, in this study they saw a decrease in the activation of antioxidant enzymes as glutathione peroxidase (GSH-Px), glutathione reductase, and glutathione S-transferase (GST), which participate in neutralizing free radicals and ROS and contribute in defending the

body against numerous disorders (Fernández-Iglesias et al., 2014). In the same regard, in a study with rats exposed to a high fat diet (HFD), which caused oxidative damage and germ cell apoptosis associated with male infertility, a treatment with GSPE could repair the oxidative damage and improved sperm quality by increasing the levels of GSH, SOD, GSH-Px and decreasing MDA production (Wang et al., 2019). Similar results were observed when male infertility was due to the exposure of mice to cadmium producing peroxidative damage in the plasma membrane of spermatozoids and resulting in a sperm dysfunction. In this case, mouse treated with GSPE showed lower levels of some oxidative stress markers as ROS, thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), nitric oxide (NO), among others, and increased the levels of GSH, tyroid-stimulating hormone (TSH) which may alter oxidative stress, vitamin C (believed to be a ROS scavenger) and vitamin E, which is the most antioxidant vitamin due to its role in radical scavenging among other antioxidant properties. (Bashir et al., 2019).

In addition to antioxidant activity, GSPE has also shown anti-inflammatory properties, playing an important role in the immune system and in other type of inflammatory processes implicated in some diseases as MetS and its comorbidities. Some *in vivo* studies carried out with GSPE in diet-induced obese rats (DIO rats), evaluated the molecular mechanisms that could explain this activity. In this regard, the potential to inhibit the formation of pro-inflammatory cytokines as Tumor Necrosis Factor-α (TNFα) in plasma, skeletal muscle, and adipose tissue, decreasing IL-6 levels in plasma and adipose tissue were some of the most important results (Terra et al., 2009, 2011; Wang et al., 2019).

Obesity and T2DM, which are the two most common comorbidities in MetS are intrinsically linked to inflammation and oxidation. In this regard, their symptoms have been shown to improve with GSPE treatment in several studies. On the one hand, there is a high amount of evidence from *in vivo* studies about obesity related to adiposity and weight loss. Specifically, it seems that proanthocyanidins extracts have proved to modulate obesity through increasing energy expenditure and suppressing food intake due to the upregulation of energy expenditure genes in the skeletal muscle and the liver, as well as decreasing fatty acid synthesis and fat uptake in the liver. Furthermore, proanthocyanidins have the ability to increase

adiponectin secretion by white adipocytes and the expression of the GLUT4 transporter in skeletal muscle too (Salvadó et al., 2015). In many studies, both using different doses of GSPE, notable changes have been observed in the improvement of obesity (Rodríguez-Pérez et al., 2019). However, it is not yet well stablished which dose is the best as there are some controversial results. For example, in two independent studies where the researchers used the same characterized proanthocyanidins grape seed extract, results from one of the studies showed that a dose of 345 mg/kg diet of GSPE during 19 weeks did not influence body weight nor adiposity index in Zucker rats fed with an HFD (Terra et al., 2009), while in the study carried out with a dose of 500 mg/kg of body weight during two weeks they found a significant decrease of body weight in DIO Wistar rats (González-Quilen et al., 2019). On the other hand, T2DM, which is closely related with obesity because there is an increased risk in insulin sensitivity when there is an excessive accumulation of visceral fat, has also been studied in several *in vivo* studies. In most of the cases, a dose of GSPE above the range of 25 to 345 mg/kg could improve insulin resistance indexes and plasma glucose and insulin levels (Pascual-Serrano et al., 2017; Rodríguez-Pérez et al., 2019; Terra et al., 2009). However, this range of doses of GSPE that are effective is widely variable among studies and further research is needed to stablish a cause-effect relationship between the GSPE administration and its effects (Rodríguez-Pérez et al., 2019).

In addition, GSPE has also an important role in decreasing the risk of cardiovascular diseases. In a study carried out in male spontaneously hypertensive rats (SHR) treated with three different doses of GSPE (250, 375 and 500 mg/kg), they found that the dose of 375 mg/kg caused a significant decrease in systolic and diastolic blood pressure. Remarkably, in the case of blood pressure the effect of GSPE was similar than the administration of captopril 50 mg/kg (Quiñones et al., 2013), which is one of the most used pharma to counteract hypertension. Hyperlipidaemia is also pointed out as one of the major CVD risks factors, referring to elevated cholesterol and tracylglycerides (TAG). In this regard, in a study in which a dose of 100 mg/kg of GSPE was administered to experimentally induced hypercholesterolemia Wistar rats a significant decrease of serum cholesterol, low-density lipoproteins (LDL), free fatty acids

(FFA) and TAG while phospholipids and high-density lipoproteins (HDL) concentration increased (Mansouri et al., 2015). A decrease in these parameters is important because when they are at high concentrations represent a great risk for the development of obstructive metabolic diseases such as arteriosclerosis and high blood pressure.

Several studies have shown that there is an important effect of GSPE in the organism's microbiota, acting as a modulator of their composition. Actually, some researchers have pointed out that maybe this is one of the mechanisms by which GSPE impacts metabolic health (Casanova-Martí et al., 2018). In the study of Casanova-Martí and collaborators, they saw that the ratio of Firmicutes:Bacteroidetes of GSPE-treated rats was lower than the ratio of the control rats and they observed differences in several taxonomic families and genera. These changes were associated with the release of different enterohormone such as glucagon-like peptide 1 (GLP-1), peptide YY (PYY), cholecystokinin (CCK) and active ghrelin, all of them involved in satiety modulation. Also, in another study carried out by Sheng and colleagues, in which they worked with an induced aged mouse model by injection of 500 mg/kg of D-galactose to evaluate the effect of GSPE on oxidative stress, inflammation levels and gut microbiota composition an improvement in oxidative stress and inflammation was observed (Sheng et al., 2022). These effects were thought to be exerted by both direct and indirect pathways due to the bidirectional communication of the gut-brain axis and gut-liver axis. In other words, these aged mice had developed gut dysbiosis which led to, on the one hand, an increase of pro-inflammatory cytokines such as TNF-a, IL-1b and NLRP3 inflammasome signalling pathways in the brain, and, on the other hand, a decrease of the anti-inflammatory cytokine IL-10 in the liver. So, GSPE modulated the composition of microbiota, and this fact had a significant influence on improving the antioxidant capacity in the liver and suppressing the inflammation in the brain.

1.3.3. Metabolism of phenolic compounds

Some authors attribute the beneficial health properties of phenolic compounds, including proanthocyanidins, to their conjugated and colonic metabolites (Bladé et al., 2016). In this regard, it is important to take into account that bioavailability of phenolic compounds depends, among other factors, on its chemical structure. This characteristic of each polyphenolic molecule is what determines its rate and extent of intestinal absorption and the natural constitution of the derived plasma metabolites (Cruz Carrión, 2021).

Figure 6. summarizes the process of digestion, absorption, distribution, metabolism, and excretion of phenolic compounds (ADME). The digestion of phenolic compounds begins in the oral cavity where various metabolic reactions take place. There, food is transformed through the chewing in which the components of the food are transformed, releasing its constituents. This is very important because phenolic compounds are found in the food with a chemical structure which is not always able to be absorbed such as esters, glycosides, or polymers. With the action of some salivary enzymes such as amylases, they are converted to aglycone forms. Once they have been hydrolysed, 5-10% of the total phenolic components are absorbed at the level of the small intestine. The remaining 90-95% reach the colon and are modified by the microbiota and further absorbed (Quiñones et al., 2012). Once absorbed, before they pass to the bloodstream, phenolic compounds are recognised as xenobiotics and its metabolization continue with the common metabolic pathway of exogenous organic substances (phase II enzymatic metabolism). In this phase, the aglycones subsequently suffer a series of modifications such as glucuronidation, sulfation and/or methylation through the corresponding enzymes (Sulfotransferases, SULs; Uridine-5'-5-diphosphate glucuronosyltransferases, UGT; and catechol-*O*-methyltransferases, COMT).

In plasma, circulating phenolic metabolites can reach different tissues and organs such as the brain, lungs, muscles, aorta, adipose tissue, spleen, among others. Moreover, levels of phenolic metabolites in plasma differ from levels in tissues (Ávila-Román et al., 2021; Cruz Carrión, 2021). Several studies have shown that different organs and tissues can continue metabolizing the phenolic compounds

due to the differential expression of several phase II enzymes (Margalef et al.,2016).

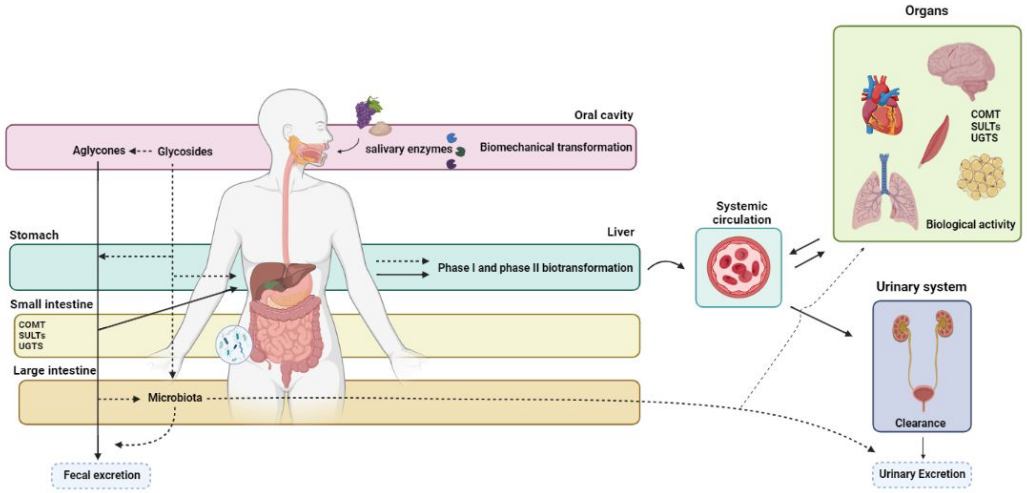


Figure 6. Overview of the ADME process for phenolic compounds. Adapted from Javier & Carrión, 2021.

1.3.4. Interaction of phenolic compounds and biological rhythms

Nutrients and bioactive compounds in food can modulate biological rhythms by changing the expression levels of different genes. In this regard, it has been described that phenolic compounds can exert modulatory effects on some genes of the clock system (Arola-Arnal et al., 2019). For instance, proanthocyanidins can act as "zeitgebers" modulating microRNAs by several biochemical and epigenetic mechanisms, thus modulating the cell functionality (Bladé et al., 2016). This function of phenolic compounds as "zeitgebers" was first evidenced in 2008 by Oike and Kobory which worked in an *in vitro* study with resveratrol and saw that was able to regulate circadian clock genes in Rat-1 fibroblast cells (Ávila-Román et al., 2021; Oike & Kobori, 2008). Since then, scientific evidence about this fact has increased. In a study in which an acute dose of proanthocyanidins was administered, nocturnal melatonin levels were altered (Ribas-Latre, Del Bas, et al., 2015). This fact was due to the ability of proanthocyanidins to modulate the oscillating expression of clock genes in the hypothalamus, where the central clock resides. Moreover, proanthocyanidins also can modulate peripheral clocks such the located in the liver (Bladé et al., 2016).

In this line of framework, related with the GSPE effect on the molecular clock machinery, Ribas-Latre and colleagues studied the expression of the clock genes in healthy and obese male Wistar Rats. Rats were treated orally with GSPE for 21 days and they saw that this treatment in healthy rats overexpressed the core clock genes both *Clock* and *Bmal1* and, the acetylated level of BMAL1, which is a SIRT1 target, also increased in the liver and the mesenteric white adipose tissue. Also, they saw a significant influence in *RORa*, *REV-ERBa*, *Nampt* and *Per2 clock-controlled genes* (Ávila-Román et al., 2021a; Ribas-Latre, Baselga-Escudero, et al., 2015). Some of these interactions are showed in **Figure 7.**

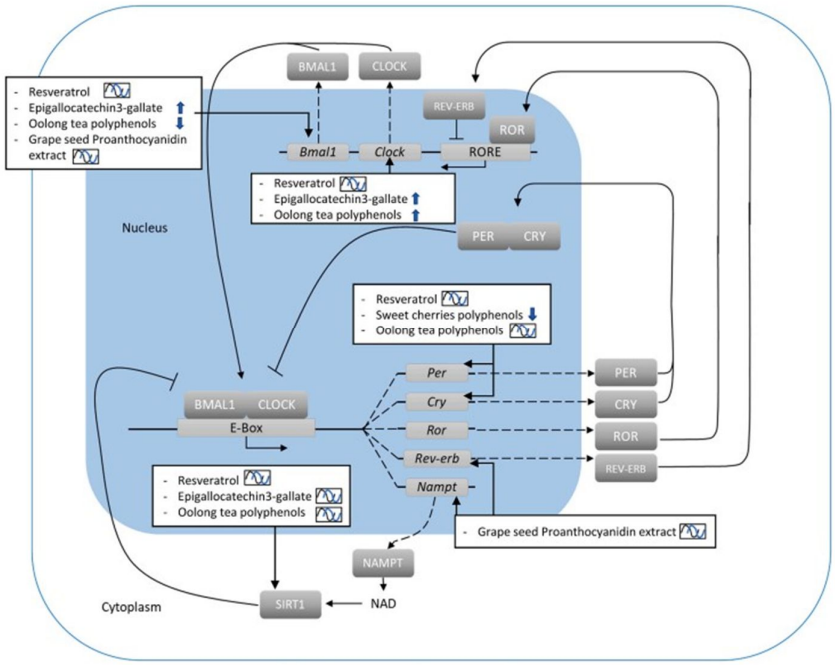


Figure 7. Influences of different phenolic compounds or its products in biologycal rhythms regulating the circadian rhythmicity in different points of intracellular machinery. (Ávila-Román et al., 2021).

1.4. Circadian desynchronization and its metabolic consequences

The chronic imbalance of circadian rhythms known as chronodisruption is produced mainly by environmental factors such as the alteration of the light-dark cycle (jetlag), shift works, the light of electronic devices at night (that can confuse the moment of the day that we are) and mutations or changes in certain genes, contributing to the development of certain chronic diseases (Johnston et al., 2016). Among these pathologies, nowadays one the most important is MetS.

Regarding to these imbalances on circadian rhythms, in several studies it has been observed that night workers had a 5% higher incidence of metabolic syndrome than daytime workers (Farha & Alefishat, 2018). Similarly, a meta-analysis carried out with nurses concluded that shift work could play a key role in the development of obesity and its related comorbidities, especially in the case of shift work at night (Zhang et al., 2020). In other recent studies in night workers, they observed an increase in some anthropometric and biochemical parameters such as a high body index, waist circumference, elevated blood pressure, TAG, total cholesterol, LDL, glycosylated haemoglobin, C-reactive protein, and low levels of HDL when it was compared with the levels presented by day shift workers (Asare-Anane et al., 2015; Ferraz-Bannitz et al., 2021). This evidence related to shift works, could be associated in some studies with common consumption of high-calorie meals at night and a greater frequency of meals and snacks, which is related to a higher risk of overweight and obesity with the consequent adverse metabolic effects (Aparicio et al., 2017).

Therefore, an adequate nutrition, where energy intake is aligned with energy expenditure, is described to help in the maintenance of circadian rhythms and physiological health (Potter et al., 2016).

1.4.1. Importance of lipid metabolism and its misalignment

In cardiovascular diseases lipids play an important role. Lipids are integral components of membrane cells and lipoproteins, and they are so important in energy storage and transport. Also, it has been seen that they play an important role as signalling molecules both in intra and intercellular level contributing to a wide range of effects in cell's physiology. It has been seen that in a

chronodisrupted environment of the organisms, its lipid profile is altered. This fact could be explained by clock-dependent regulation of lipids species. If circadian clock is disrupted, the lipid metabolism is desynchronised and this is associated with the onset of obesity and metabolic disease (Gooley, 2016).

Different clock genes are involved in lipid metabolisms. For instance, in the liver it has been seen that PGC1a is upregulated by the PPARa, a clock regulated gene, and it has an important role coordinating the circadian function and energetic metabolism. PGC1a can exert an effect against the ROR family members, and this interaction is required for the functionality of the cell-autonomous clock, coordinating the circadian clock function and energy metabolism. In the skeletal muscle, most of the transcripts regulated circadianly are referred to different lipid pathways. It has been found some nuclear receptors and their co-regulators, genes involved in fatty acid oxidation, lipid synthesis and hydrolysis of TAG. The lipid absorption in the enterocytes has also a daily regulation by the CLOCK:BMAL transcriptional complex which activates the small heterodimer partner (Shp) which its protein have the ability to supress the microsomal TAG transfer protein (Mtp). In the small intestine, the enzyme deadenylase nocturnin (CornYl) is necessary for the absorption and secretion of lipids. Finally, in the adipose tissue, especially the white adipose tissue (WAT), it has been seen that adipogenesis and lipolysis are strongly regulated by certain clock genes (Gooley, 2016). On the one hand, it has been described that BMAL1 is a positive regulator of adipogenesis in preadipocytes (Shimba et al., 2005). On the other hand, it has also been described that BMAL1 has totally opposite properties and it was seen to inhibit the differentiation of stem mesenchymal cells (Guo et al., 2012). However, nowadays it is believed that BMAL1 has a biphasic role in which in primarily states inhibits the stem cells differentiation but lately it promotes lipogenic processes (Kiehn et al., 2017). Furthermore, in *in vivo* studies it has been seen that mutations in *Bmal1* are correlated with an increase in adiposity. Also, during the resting and fasting phase, when the organism's energy demand is compensated with the activation of the lipolysis in WA, TAG starts to rhythmically release FFA and glycerol in the bloodstream. This fact could be explained by the rate-limiting enzymes in the lipolysis pathway, which are BMAL1 targets. Among these enzymes it can be found the adipocyte TG lipase (ATGL)

and hormone-sensitive lipase (HSL). Also, triglyceride hydrolase (TGH), which is the major hydrolase enzyme contributor in lipid release in WAT is rhythmically expressed because it is thought to be a direct target of adipose heterodimer CLOCK/BMAL1 regulation (Kiehn et al., 2017).

1.4.2. Blood pressure rhythmicity

In addition to the direct consequences that produce high levels of lipids in plasma, this is strongly related with the increase in the blood pressure and the development of several non-communicable diseases (P. Larochelle, 2002).

It has been described that blood pressure has a circadian oscillation through the day, showing a reduced values during sleep and increased ones during the awakening phase. Therefore, during each day-time cycle, it has been seen two daytime peaks in which the first is around the commencement of day approximately in the hours comprised with 8:00-10:00 and a second, which has a smaller magnitude, taking place between the 19:00-21:00. On some occasions, there is a small afternoon nadir, generally in 15:00. This blood pressure rhythmicity is because several processes that regulate the blood pressure have their own endogenous circadian rhythms. Among these events outstand the melatonin release and the renin-angiotensin-aldosterone system (RAAS) (Fabbian et al., 2013; Izzedine et al., 2006).

In normal conditions, during the transition from waking up to sleeping, the blood pressure declines on average by 10-20%. This phenomenon is known as “dipping” and the people who present this decrease in its pattern are known as “dippers”. However, it is described that in some individuals this nocturnal fall of the blood pressure is blunted or lost. They are known as “non-dippers”. Furthermore, there is another classification in which there are subjects that its nocturnal blood pressure continues rising and achieving values similar to the ones common during the wake-time and they are called “risers” (Fabbian et al., 2013). This loss of rhythmicity has been associated with metabolic disorders, progression of chronic kidney disease and contributing to the development of cardiovascular diseases (Rhoads et al., 2020).

1.4.3. Influence of circadian rhythm on oxidative stress enzymes

The disruption of circadian rhythms also leads to an imbalance in the homeostasis of the free radical levels, promoting a pro-oxidant environment that is involved in the development of various diseases due to the oxidative damage. (Budkowska et al., 2022; Fernández Iglesias, 2013). Free radicals are the product of mitochondrial respiratory activity and, in fact, 1% of the total oxygen that mitochondria use to carry out respiration is destined to the generation of ROS. For the elimination of these species, the body has several antioxidant defence mechanisms either enzymatic, including SOD, CAT, GPx, GST, or non-enzymatic, including glutathione (GSH), vitamins (C and E), carotenoids, and flavonoids, among others.

It has been seen that one of the most important roles of the dormancy period of organisms is to reduce the number of free oxygen species generated throughout the day in order to restore their basal levels. This fact is explained by the antioxidant role of melatonin. That is why an imbalance in the circadian rhythm of organisms leads to serious health problems as has been observed in different studies related to shift work (Hohor et al., 2023) and in animals in different models of disruption in which they had lower levels of SOD, CAT (Villafuerte et al., 2015). Furthermore, it has been reported that several antioxidant enzymes such as SOD and CAT are regulated by the CLOCK and BMAL1, but the mechanism remains unclear (Budkowska et al., 2022).

2. HYPOTHESIS AND OBJECTIVES

Nowadays societies are subjected to a great diversity of factors that end up inducing alterations in biological rhythms, with a plethora of serious health consequences such as the development of different metabolic diseases. Among these factors, previously mentioned in more detail, are mutations or changes in certain genes, schedule mismatches or shift work, such a high intake of snacks and unhealthy food products and light from electronic devices at night can confuse biological clocks.

Currently, there are no effective strategies to avoid these rhythm disorders, or the negative effects caused by the desynchronization of biological clocks. However, it is known that the different bioactive compounds present in the diet, such as phenols, can play an essential role in the modulation of these rhythms.

It is for all these reasons that, given the evidence that exists regarding circadian rhythms and phenolic compounds, our **hypothesis is that the chronic consumption of GSPE can reverse or alleviate the appearance of metabolic alterations produced as a consequence of the disruption of the circadian rhythm**.

In order to verify or ratify our hypothesis, two different **main objectives** were proposed:

- To study the **changes** that occur in an animal model that has an **altered circadian rhythm** at different levels.
- To evaluate the **ability of GSPE** to **prevent** or **alleviate** the symptoms generated as a result of the disruption of the circadian rhythm in this animal model.

In addition, to achieve the previously mentioned main objectives, three **secondary objectives** were suggested:

- First, to analyse the **biometric markers** including body weight, food intake, tissue weights and blood pressure.
- Second, to evaluate the **biochemical plasma concentrations** of glucose, cholesterol, TAG, HDL, LDL, VLDL, NEFAs, phospholipids and glycerol.

- Finally, to study the **hepatic oxidative stress** determining the catalase activity and the MDA concentration.

3. MATERIALS AND METHODS

3.1. Animals and experimental design

This study was conducted with 24 healthy 12-week-old adult male Wistar rats grouped into three experimental groups (n=8). The animals were housed individually under different light-dark cycle conditions. The stabled housing conditions were 22 ºC temperature and 55% of humidity. They were fed *ad libitum* with a standard chow diet (Tekland global 14% protein rodent maintenance diet, Envigo) and water. After a period of adaptation, rats were operated to implant a HD-S10 sensor (Data Sciences International) in the animal's abdominal aorta. This telemetric system allows to continuously register different rhythmic variables of the animals as body temperature, blood pressure (diastolic, systolic, and total), and the activity-rest alternation without the need to handle them. After surgery, animals were given two weeks of recovery and then the experimental phase began. Additionally, body weight and food intake were monitored weekly during the study.

The three groups of rats were subjected to different light-dark cycles (**Figure 8.**). On the one hand, those rats belonging to the control group were subjected to symmetrical cycles of 24 hours duration (12h light/12h dark). The other two groups were subjected to symmetrical light-dark cycles of 22 hours of duration (11h light/11h dark) as an experimental model of chronodisruption. One of the 11h/11h group was treated with GSPE in a dose equivalent to 25 mg/kg/day. The other 11h/11h group and the control group were treated with a vehicle (water). Both GSPE and the vehicle were ingested by voluntary licking for 8 weeks.

After 8 weeks of treatment, all the animals were sacrificed by decapitation 2 hours after the last treatment. At that moment, the volume and weights of whole blood, liver, muscle, and brain were collected and measured, quickly frozen afterward, and stored at -80 ºC until further analysis. The blood was collected with the corresponding Vacutainer®, and it was centrifuged at 3,000 g for 15 minutes (Eppendorf centrifuge 5804 R) to obtain plasma before it was stored. Furthermore, the different deposits of adipose tissue, including the mesenteric (MWAT), epididymal (EWAT), inguinal (IWAT), brown (BAT), perirenal fat, and

subcutaneous tissue, were excised and weighed separately to calculate the adiposity index (AI).

Animal experiments were approved by the Animal Ethics Committee of Universitat Rovira i Virgili and were carried out in accordance with the correspondent directive of the Council of the UE and the procedures established by the Departament d'Agricultura, Ramaderia i Pesca of Generalitat de Catalunya (Barcelona, Spain). In addition, the rats were manipulated by the technicians and PhD students, qualified personal with the suitable experimental animal course and when this bachelor's project was done, this experimental method had already finished.

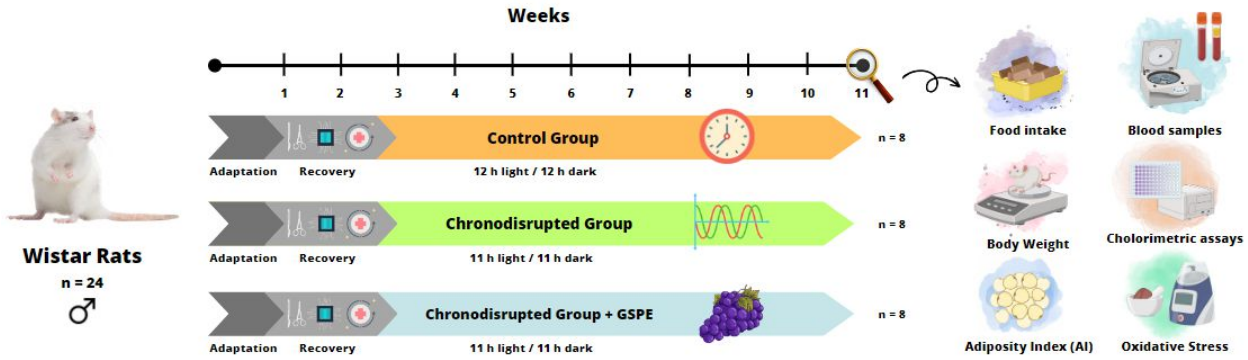


Figure 8. Summary of the experimental design with animals

3.2. Grape Seed Proanthocyanidins Extract (GSPE)

The grape seed proanthocyanidins extract (GSPE) used in this study was composed of monomers (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%), and oligomers (5-13 units; 31.7%) of proanthocyanidins according to the manufacturer (Les Dérivés Résiniques et Terpéniques, Dax, France. The phenolic composition of this extract, shown in **Table 1**, was analysed by Margalef *et al.* by HPLC-ESI-MS/MS. (Margalef et al., 2015).

Table 1. Main Phenolic Compounds of the GSPE used analyzed by HPLC-ESI-MS/MS (Margalef et al., 2015).

Compound	Concent. (mg/g)	Compound	Concent. (mg/g)
Gallic acid	31.07 ± 0.08	**Epicatechin**	93.44 ± 4.27
Protocatechuic acid	1.34 ± 0.02	**Dimer gallate**	8.86 ± 0.14
Vanillic acid	0.77 ± 0.04	**Epicatechin gallate**	21.24 ± 1.08
PA dimer B2	33.24 ± 1.39	**Epigallocatechin gallate**	0.03 ± 0.00
PA dimer B1	88.80 ± 3.46	**Epigallocatechin gallate**	0.27 ± 0.03
PA dimer B3	46.09 ± 2.07	**PA trimer**	4.90 ± 0.47
Catechin	121.32 ± 3.41	**PA tetramer**	0.05 ± 0.01

Abbreviations: PA (proanthocyanidins). The results are expressed on a wet basis as the mean ± SD. The results are expressed as mg of phenolic compound per g of GSPE.

3.3. Biometric Parameters

Several biometric parameters, including cumulative body weight gain, food intake, systolic blood pressure, and adiposity index (AI) were calculated and evaluated. Cumulative body weight gain was calculated by taking the rat's weekly weight minus their weight in the first week of the experiment. Food intake was calculated considering the food that was ingested by the rats during the day and night, separately. Systolic blood pressure was weekly registered with the HD-10 sensor.

After the rats were sacrificed, different organs such as the liver, brain, leg muscle (gastrocnemius and soleus), cecum, and mesenteric (MWAT), epididymal (EWAT), inguinal (IWAT), brown (BAT), perirenal, and subcutaneous adipose depots, were excised and weighed to see the differences between the three groups.

AI was calculated by the sum of all the adipose depots divided by the total body weight (g) and it was expressed as a percentage of adiposity (Bastías-Pérez et al., 2020).

3.4. Circulating Biomarkers in Plasma Samples

Analysis of plasma circulating parameters were performed to obtain information on the general metabolic situation of the animals. Total cholesterol (TC), TAG, and glucose were determined with colorimetric enzymatic kits commercialized by QCA (Amposta, Tarragona, Spain). Cholesterol-HDL determination was performed with other colorimetric enzymatic kits from the Biosystems commercial house (Barcelona, Spain). LDL and very low-density lipoprotein (VLDL) fractions were calculated according to the Friedewald equation (LDL =TC– (HDL + VLDL); VLDL = TAG/5) (De Oliveira et al., 2019). The enzymatic kits for phospholipids determination were supplied from SpinReact (Barcelona, Spain), non-esterified fatty acids (NEFAs) were from Fujifilm (Neuss, Germany), and the used for glycerol was from Sigma Aldrich (USA). All the determinations done with kits were carried out in 96-well plate following the instructions of the manufacturers and read using an Eon BioTek spectrophotometer (Izasa, Barcelona, Spain) expressing the results as mg/dL.

Different indices were calculated to know the cardiovascular risk and, therefore, the state of health of the animals. Among those, we calculated the Castelli index I and Castelli index II, the atherogenic index (AIP), the atherogenic coefficient (AC), and the lipid fraction without considering HDLc (NHc). The equations used to calculate the indices were: Castelli index I = TC/HDLc; Castelli index II = LDLc/HDLc; AIP = Log (TAG/HDLc); AC = (TC – HDLc)/HDLc; Non-HDLc (NHC) = TC – HDLc (De Oliveira et al., 2019; Ruiz de Azua.J, 2022).

3.5. Oxidative Stress Biomarkers

To see the oxidative stress status of the animals, the hepatic MDA concentration and the CAT activity were analysed. On the one hand, MDA levels were determined in the liver by the TBARS assay. For this determination, it was first prepared a liver homogenate using phosphate buffer (pH 7, 30 mM) following a proportion of 1:9 (liver: phosphate buffer). The liver was weighed, and the phosphate buffer was added to a 2 mL eppendorf with a metallic bead, and it was put into the Qiagen TissueLyser (Hilden, Germany). Then, 200 mL of homogenate were added with 300 mL of KCl 0.15 M, 1.5 mL of H3PO4 1%, and 500 mL of thiobarbituric acid 30 mM and it was incubated in a 100 ºC bath for 1

hour. After incubation, it was added 2 mL of butanol and it was centrifuged for 20 minutes at 3,000 g. Finally, the absorbances were read in three different wavelengths (305, 332, and 356 nm) with a Hitachi U-1800 spectrophotometer (Berkshire, UK). The same protocol was used to obtain the standard curve employing an 8.26 mM 1,1,3,3-Tetraethoxypropane (TEP) solution at different concentrations. On the other hand, CAT activity was assayed measuring the disappearance of hydrogen peroxide using spectroscopy. For the determination, the supernatant obtained from the liver homogenate previously done for the MDA determination was used. The homogenate was centrifuged for 15 minutes, at 4 ºC and 3,000 g (Hettich Zentrifugen Mikro 200R, Germany) and it was diluted 1/100 in the same phosphate buffer in which the homogenate was prepared. The absorbance was read doing a kinetics during one minute in a wavelength of 240 nm with the Hitachi U-1800 spectrophotometer (Berkshire, UK). First, the sample was put to a quartz cuvette and the blank was read. Then, it was put immediately into the cuvette a 30 mM hydrogen peroxide solution to start the reaction.

The oxidative stress results were normalized by the amount of hepatic protein of the sample. To determine the protein concentration, it was used the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Barcelona).

3.6. Statistical Analysis

Statistical tests and the graphics were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant. Data were analysed using two-way analysis of variance (ANOVA), one-way ANOVA or the Kruskal-Wallis test, if the data was non-parametric. To determine if the values were parametric or non-parametric, it was first tested the normality using the Shapiro-Wilk test, followed by Barlett's test which is equivalent to Levene's test to analyse the homoscedasticity of the values in GraphPad. For the lipid profile analysis, it was used the Metaboanalyst 5.0 platform was used using all the lipid parameters previously determined.

4. RESULTS

4.1. Effect of circadian desynchrony on biometric parameters

4.1.1. Body weight

Figure 9. shows body weights of the rats at an initial point. As it can be seen, data did not present significant differences between them, confirming that all the animals used in the experiment enrolled in the experiment in the same conditions of corporal weight.

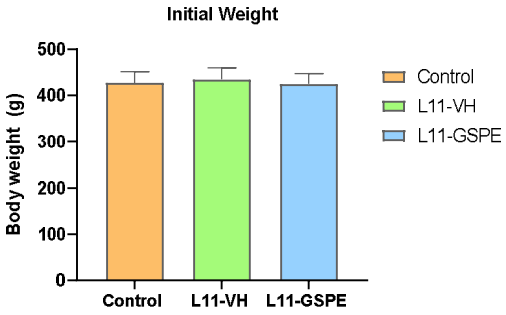


Figure 9. Mean value and SEM of rat's body weight (g) at the beginning of the experiment.

After this consideration, **Figure 10.** shows the data obtained from the cumulative body weight gain during the 8 weeks of the experiment. Since the first week, a significant difference in cumulative body weight can be observed between the disrupted groups and the control group. The control group had higher values of cumulative body weight than the chronodisrupted groups.

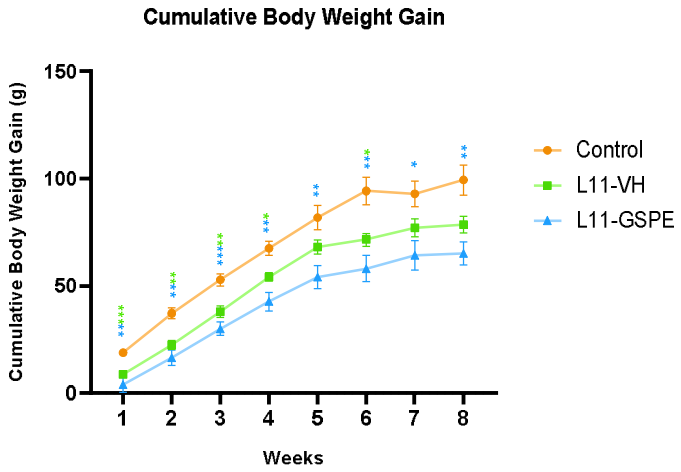


Figure 10. Cumulative body weight gain during the eight weeks of treatment in every group. 2-way ANOVA Repeated Mesures and Post-hoc one-way ANOVA for treatments in every week, Tukey's multiple comparisons vs control. *p<0.05 **p<0.01 ***p<0.001. The significant differences shown are between the disrupted groups vs control according to the legend.

Also, the evolution of the body weight of the animals is represented in **Figure 11**. In this case, the significant statistically differences were lost. It should be noticed that, in this graph there are represented nine weeks instead of eight. This is because the first point represents the adaptation week.

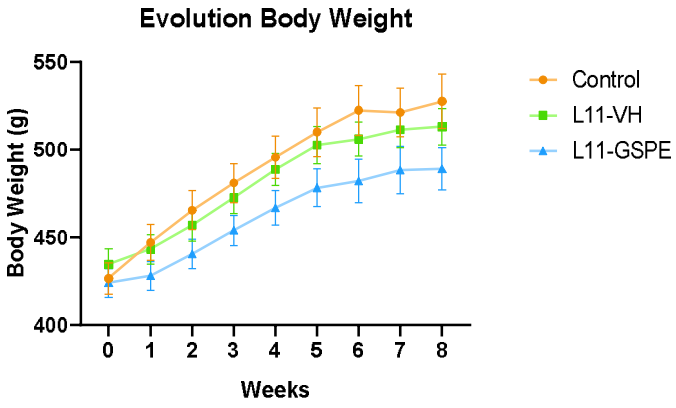


Figure 11. Evolution of body weight during the eight weeks of treatment in every group. 2-way ANOVA Repeated Mesures and Post-hoc one-way ANOVA for treatments in every week, Tukey's multiple comparisons vs control.

4.1.2. Food intake

Figure 12. shows the total food intake by the rats. The measures represent the sum of the food ingested during the day and the food ingested (g) during the night with its SEM value. It could also be seen significant differences in the third, sixth, and seventh weeks between the control group and the disrupted one treated with GSPE.

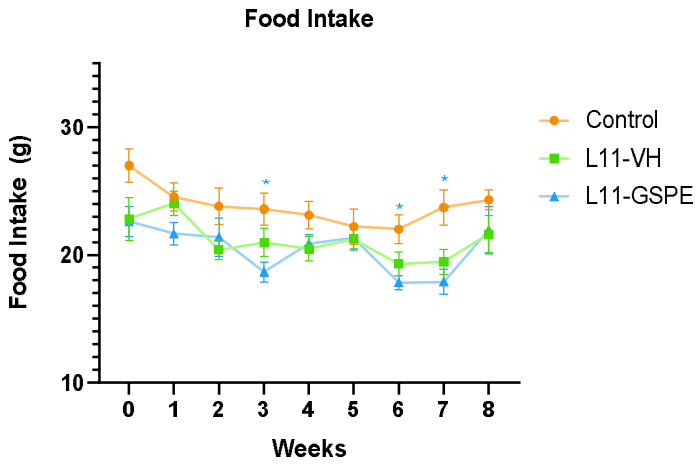


Figure 12. Evolution of body weight during the eight weeks of treatment in every group. 2-way ANOVA Repeated Mesures and Post-hoc one-way ANOVA for treatments in every week, Tukey's multiple comparisons vs control. *p<0.05 L11-GSPE vs control.

In order to study in more depth the dietary pattern of these animals, we evaluated the differences between the food intake of the animals during the light and dark phase. Data are represented in **Figure 13**.

On the one hand, the food intake of the control group during the light phase, which is the rest period for the rats, was lower compared to the two chronodisrupted groups. On the other hand, the food intake during the night phase, which is the rat's activity period, was higher in the control group compared to L11-VH and L11-GSPE groups. Furthermore, it could be seen that, in the food intake during the light phase there were more statistically differences than during the dark period.

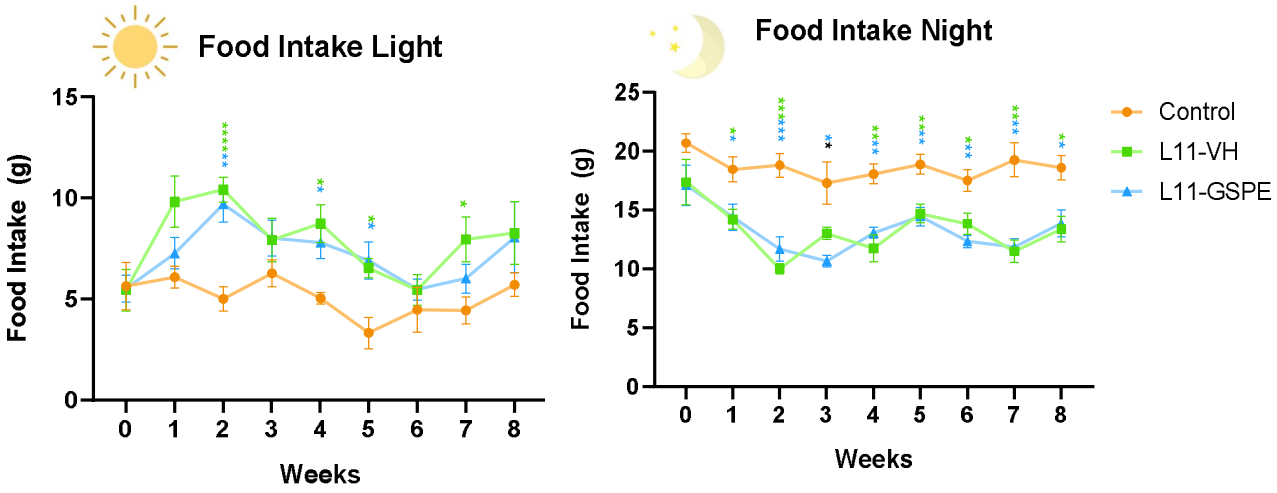


Figure 13. Food intake evaluation during the light and dark phase in every group. 2-way ANOVA Repeated Mesures and Post-hoc one-way ANOVA for treatments in every week, Tukey's multiple comparisons vs control. *p<0.05, **p<0.01 ***p<0.001. The significant differences shown are between the disrupted groups vs control according to the legend and the black * shows the statistically significant difference between the L11-VH and L11-GSPE groups.

4.1.3. Systolic blood pressure

Figure 14. shows data from systolic blood pressure (SBP, mmHg) through a period of 48 hours. From the hour 1 to hour 12, represents the phase in which the lights were switched on and from hour 13 to hour 24 the dark phase, repeating that another light-dark cycle until hour 48 (except for the first week, in which the data was recorded only during the first 24 hours). In the case of the disrupted groups, it is represented in the same way but, comprising light/dark periods of 11

hours, with the first light cycle being from hour 1 to hour 11 and the first dark cycle from hour 12 to hour 22 and so on until hour 44.

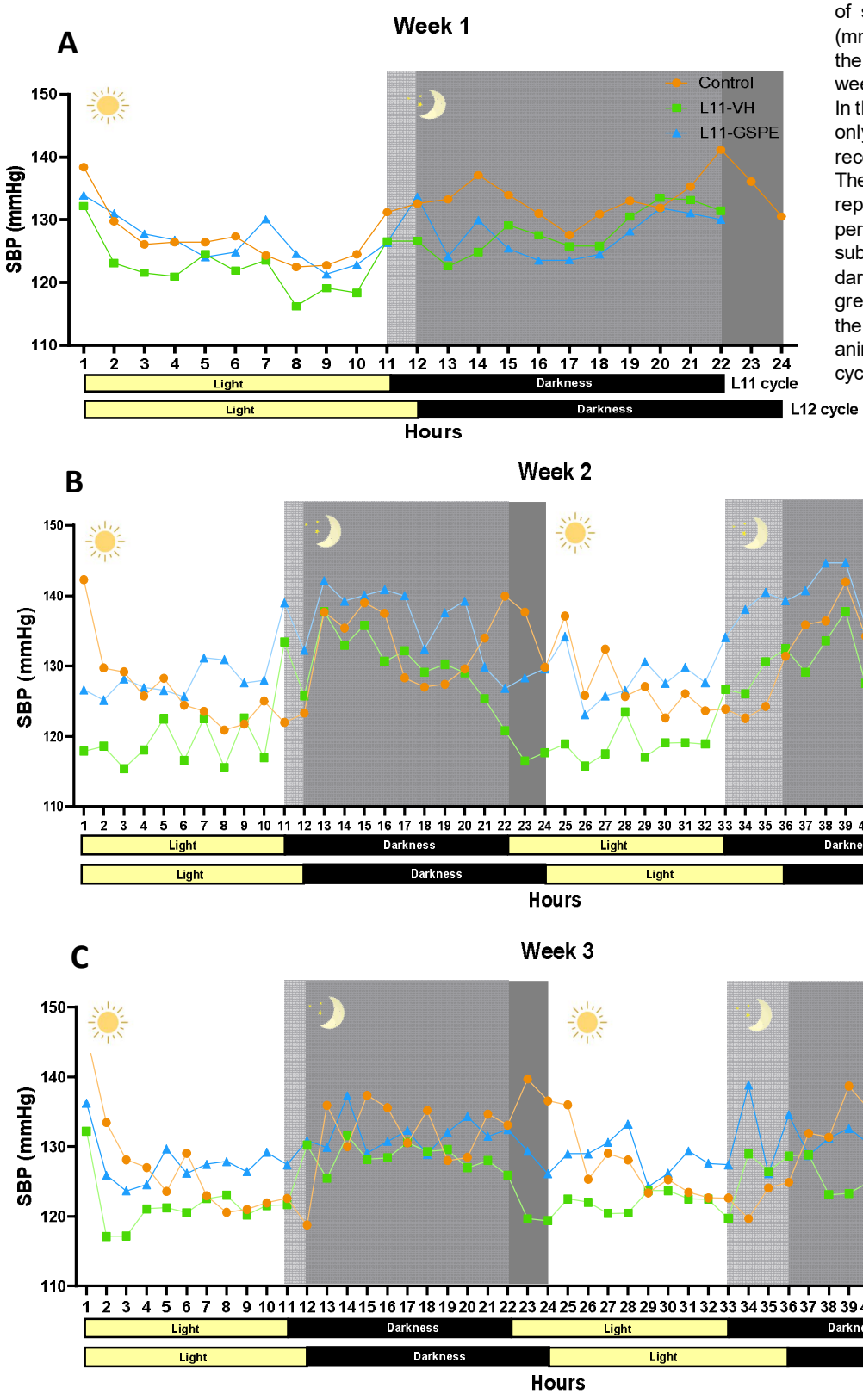
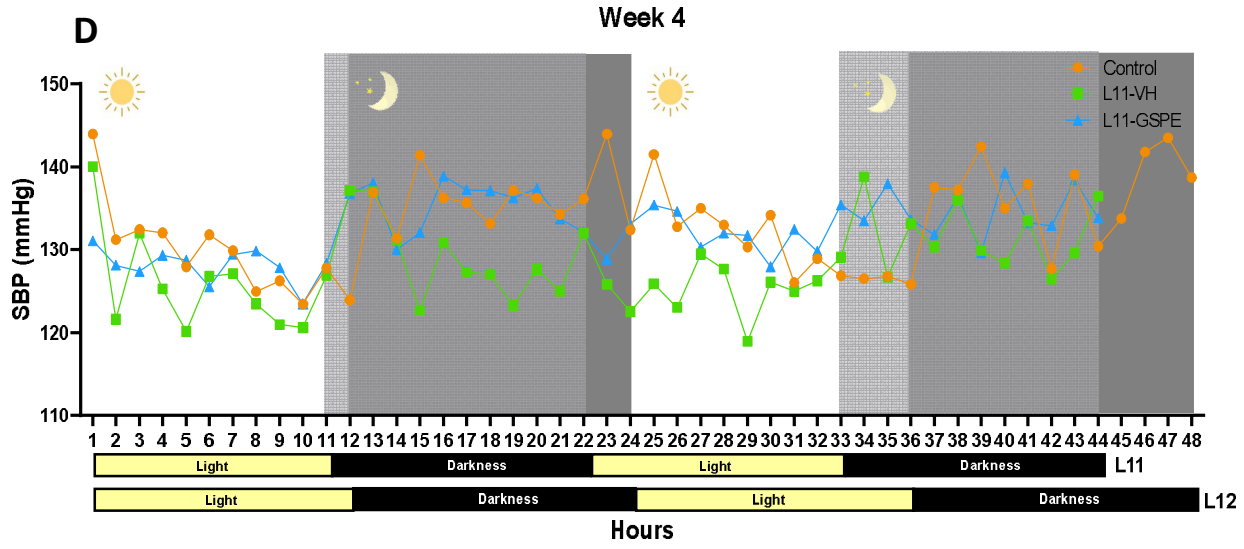
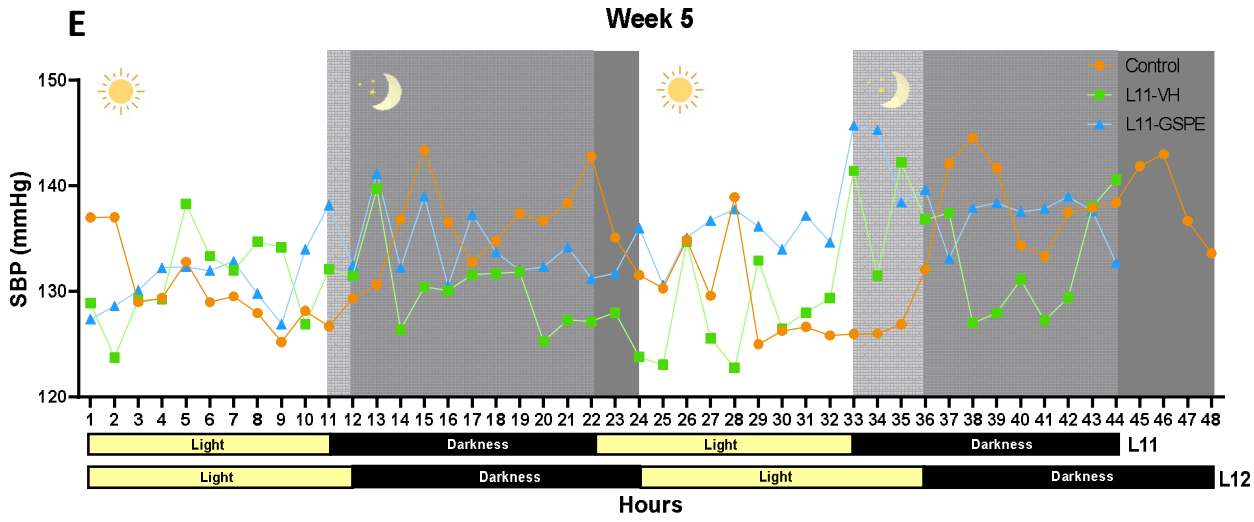
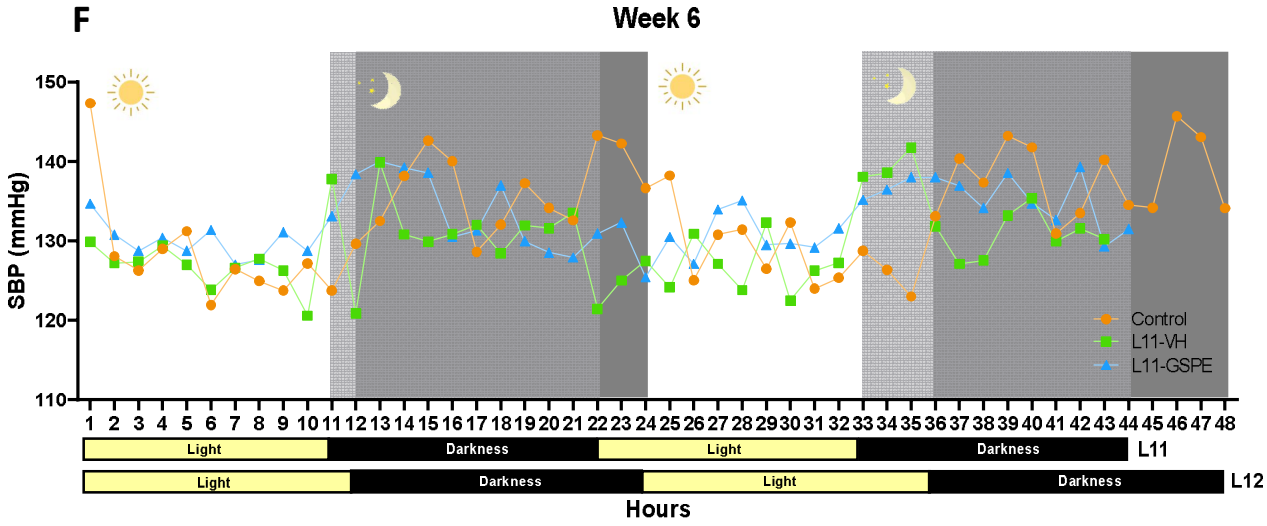
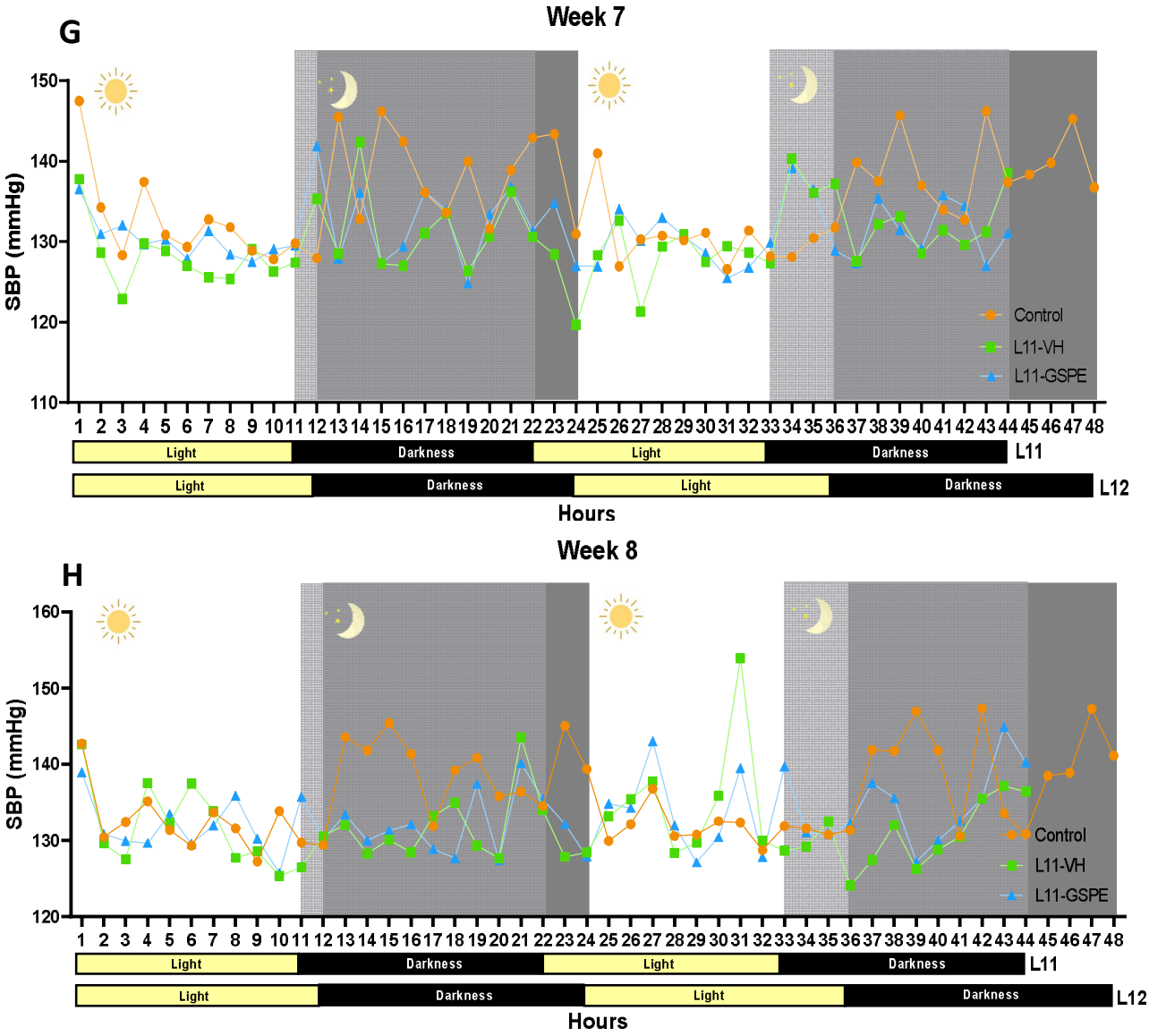


Figure 14. Representation of systolic blood pressure (mmHg) variying through the time in the different weeks (A, B, C, D, E, F, G). In the first week (A) there is only showed the data recorded during 24 hours. The light grey shadow represents the darkness period of the animals submitted to a 11h light-dark cycles while the dark grey shadow represents the darkness period of the animals with 12 h light-dark cycles.


D
Week 4
SBP (mmHg)
150
140
130
120
110
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Control
L11-VH
L11-GSPE
Light
Darkness
Light
Darkness
L11
Light
Darkness
Light
Darkness
L12
Hours


E
Week 5
SBP (mmHg)
150
140
130
120
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Control
L11-VH
L11-GSPE
Light
Darkness
Light
Darkness
L11
Light
Darkness
Light
Darkness
L12
Hours


F
Week 6
SBP (mmHg)
150
140
130
120
110
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Control
L11-VH
L11-GSPE
Light
Darkness
Light
Darkness
L11
Light
Darkness
Light
Darkness
L12
Hours



Abbreviations: SBP (Systolic Blood Pressure), L11 (11 h light – 11 h dark cycle), L12 (12 h light- 12 h dark cycle). A: SBP data refered to week 1; B: SBP data refered to week 2; C: SBP data refered to week 3; D: SBP data refered to week 4; E: SBP data refered to week 5; F: SBP data refered to week 6; G: SBP data refered to week 7; H: SBP data refered to week 8.

In the first week (**Figure 14.A**), a homogeneous pattern could be observed between the three groups. There were no excessive blood pressure peaks and all groups followed the same pattern. In the second week (**Figure 14.B**) some changes in the systolic blood pressure peaks began to be seen, sometimes acquiring a higher systolic blood pressure in the chronodisrupted groups compared to the control group but, it could not be said that the chronodisrupted groups had hypertension because it is the only week that this event happened. From this moment on, a loss of rhythmicity was observed, and this fact got worse

over the weeks. In other words, regarding the representation of the systolic blood pressure of the control group, a blood pressure pattern of down-up-down-up was clearly observed. This same pattern is maintained more or less in the chronodisrupted groups until week 4 (**Figure 14.D**). From this week onwards, no type of rhythmicity can be observed in either of the two chronodisrupted groups. Furthermore, in week seven and eight it could be observed that systolic blood pressure follows an almost linear pattern.

4.1.4. Tissue weights

Figure 15. shows the graphs referring to the average weights of the organs of the animals belonging to the different groups.

First, regarding the liver's weight (**Figure 15.A**), no significant differences were observed between the three groups, with average values for the control group of 11.15 ± 0.53 g, for the L11-VH group of 12.06 ± 0.40 g and by the L11-GSPE group 11.71 ± 0.43 g.

Second, regarding the average weights of the brain (**Figure 15.B**), no significant differences were observed between the three groups, reaching values of 2.09 ± 0.03 g in the control group, 2.00 ± 0.04 g in the L11-VH group and 1.99 ± 0.03 g in the L11-GSPE group.

Furthermore, in the muscle (**Figure 15.C**), which is the total weight of the gastrocnemius and soleus muscles, no significant differences were observed between the average weights either, taking values of 1.27 ± 0.09 g in the control group, 1.30 ± 0.05 g in the L11-VH group and 1.21 ± 0.03 g in the L11-GSPE group.

Finally, regarding the weights of the cecum (**Figure 15.D**), although there were no significant differences between the weights, a greater value was observed in the group of disrupted rats with 6.37 ± 0.65 g compared to the control 5.00 ± 0.22 g. In addition, the weight of the cecum in the group of disrupted rats treated with GSPE was slightly lower than in those disrupted without treatment although a clear effect of GSPE was not observed, not reaching significant differences.

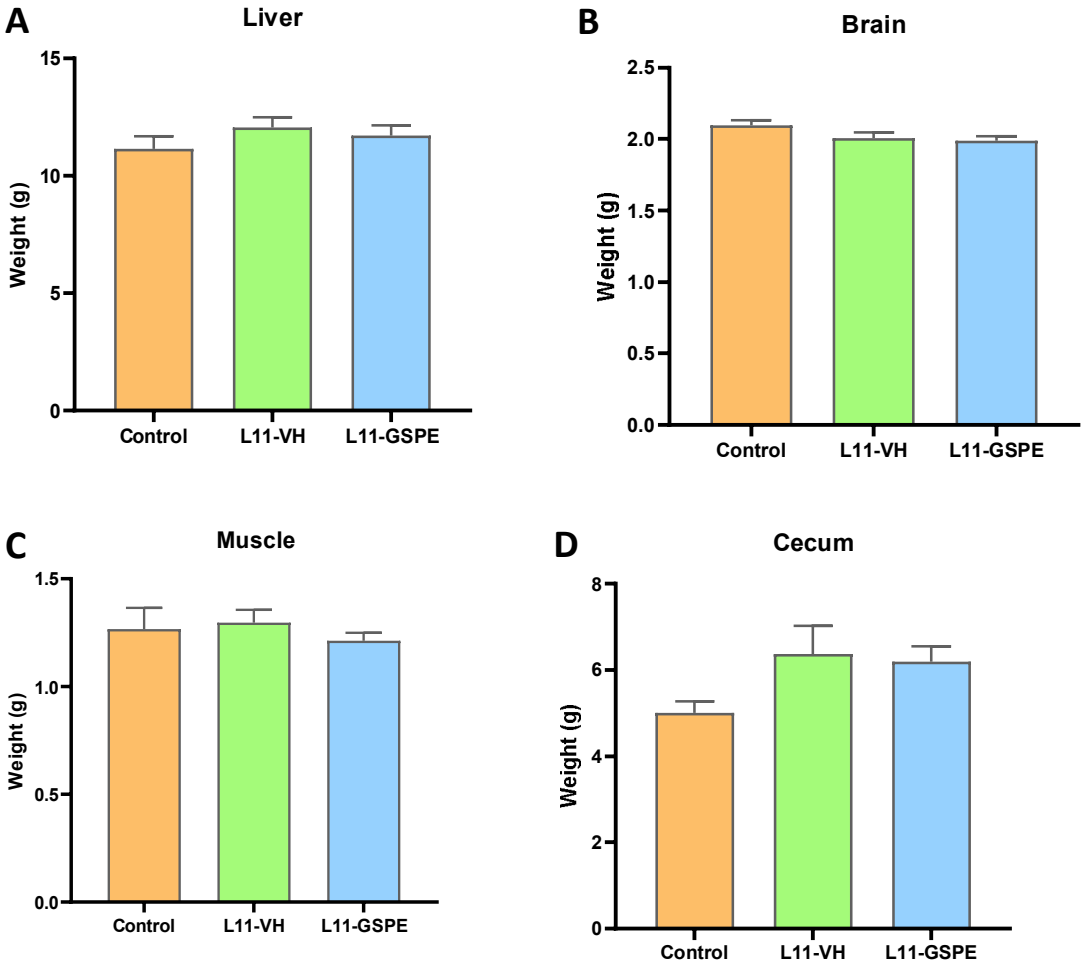


Figure 15. Representation of the mean weights of different organs from each experimental group with its SEM value. Kruskal-Wallis test and Post-hoc analysis for treatments, Dunn's múltiple comparisons vs control.

Regarding the adipose tissues depots (**Figure 16.**), in the mesenteric (**Figure 16.A**), which is the adipose tissue that attaches around the different segments of the intestines, no significant differences were observed in any of the three groups, obtaining mean weight values of 5.27 ± 0.58 g in the control group, 5.47 ± 0.40 g in the disrupted group with vehicle and 6.15 ± 0.43 g in the disrupted group treated with GSPE.

In the epididymal adipose tissue (**Figure 16.B**), which is located in the gonadal adipose tissue between the testis and the head of the epididymis, no significant differences were observed between the three groups, with values of 8.05 ± 0.83 g in the control group, 9.51 ± 0.83 g in the L11-VH group and 7.08 ± 1.19 g in the L11-GSPE group.

In the inguinal adipose tissue (**Figure 16.C**), which spreads from the dorsolumbar region to the gluteal region, despite the difference not being statistically significant, a clear increase in the weight of this deposit was observed in the group L11-VH with an average value of 4.53 ± 0.70 g compared to the control group with 2.35 ± 0.30 g. On the other hand, it seems that the disrupted group with GSPE treatment reduced the weight of the adipose depot approaching to the value obtained in the control group and being lower than that of the disrupted treated with vehicle, being the average value of this tissue of 3.35 ± 0.82 g.

Regarding the brown adipose tissue (**Figure 16.D**) which has an important function in the oxidation of lipids to produce heat, although not statistically significant difference was observed, it was smaller in the L11-VH group (0.36 ± 0.02 g) compared to the control group (0.54 ± 0.09 g) and the L11-GSPE group (0.42 ± 0.04 g).

Interestingly, the perirenal fat (**Figure 16.E**), which is a component of the visceral adipose tissue that surrounds the kidneys with a very important metabolic activity, presented significant differences in weight between the L11-VH group with 8.96 ± 0.75 and the L11-GSPE group with a value of 7.42 ± 1.45 g. The weight of L11-VH group did not present a statistically significant difference compared to the control group with 6.57 ± 0.52 g despite also being a lower weight value as L11-GSPE had. This result suggested that the L11-VH group, presented a greater amount of perirenal fat than the control and a statistically significant greater weight than the L11-GSPE group.

Finally, in the subcutaneous tissue (**Figure 16.F**) a clear significant difference was observed between the control group (9.84 ± 0.89 g) and the L11-VH group (14.18 ± 1.34 g) while in the L11-GSPE group, despite the difference not being statistically significant, the value was close to the control group (10.26 ± 0.69 g).

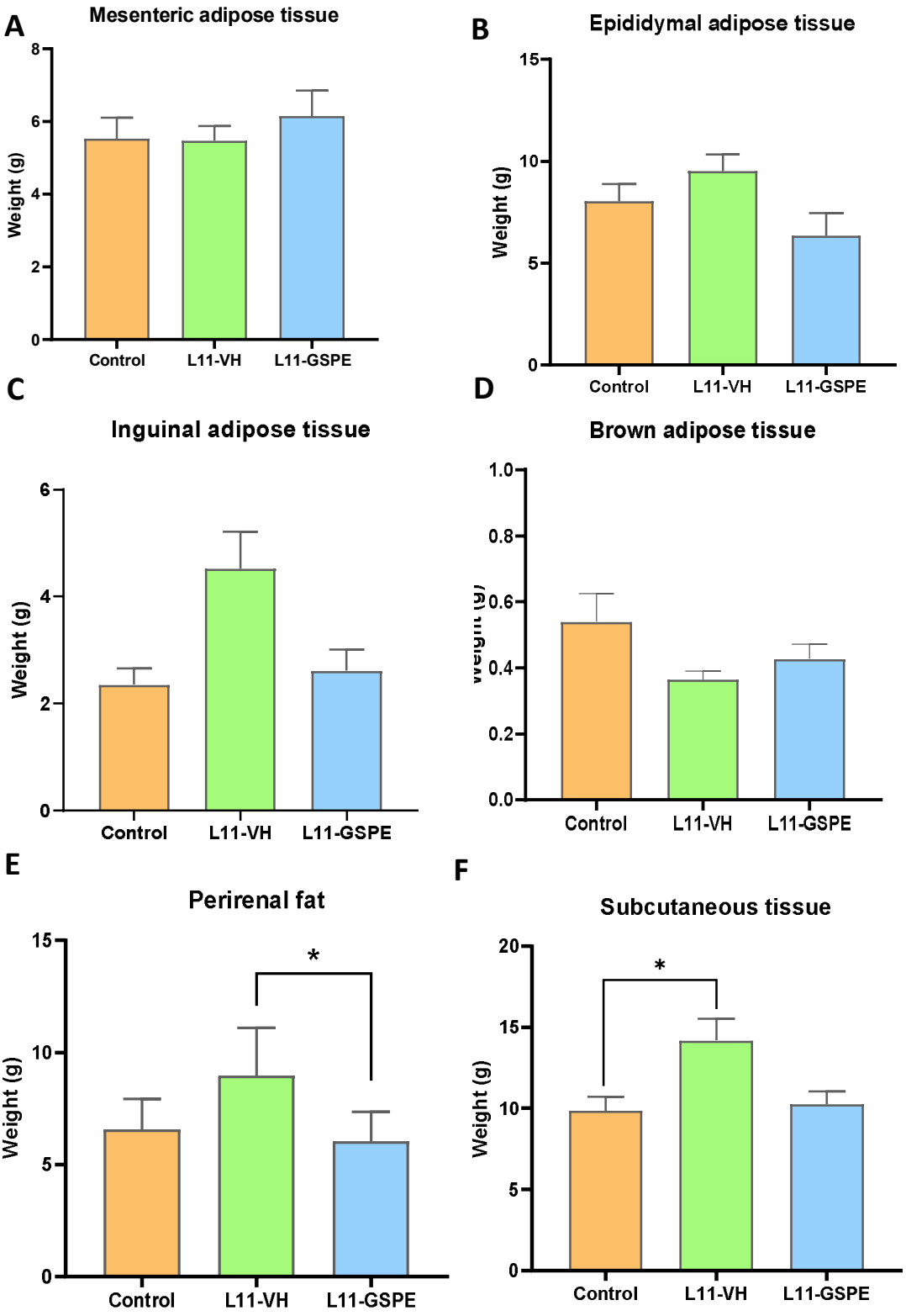


Figure 16. Representation of the weights of different adipose depots from each experimental group. Kruskal-Wallis test and post-hoc analysis for treatments, Dunn's multiple comparisons vs control. *p<0.05.

With the data obtained from the different adipose tissues the adiposity index was calculated for each group. The weights of all adipose tissues were added up and divided by the total weight of each animal and multiplied by 100, thus expressed as a percentage. **Figure 17.** shows the graph corresponding to the average values obtained through this calculation with its corresponding standard deviation.

A significant difference was observed between the control group and the L11-VH group while, compared with the L11-GSPE group no significant differences were observed. However, the value presented by this last group tended to be more similar to the control group.

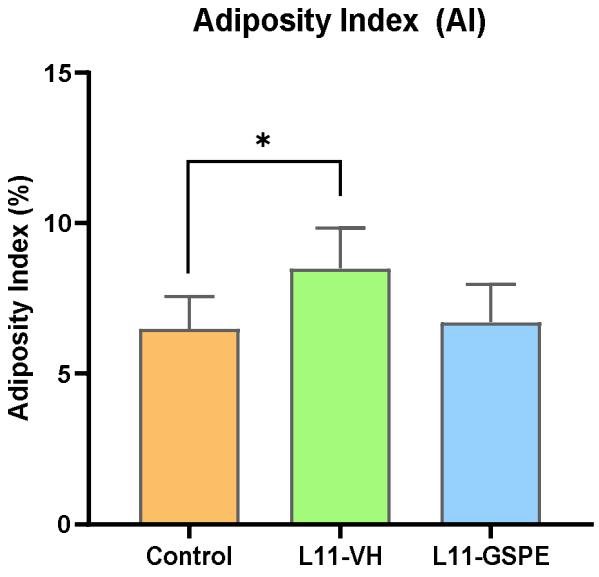


Figure 17. Representation of the adiposity index (AI) for each experimental group with the data of adipose tissue depots. Kruskal-Wallis test and post-hoc analysis for treatments, Dunn's multiple comparisons vs control. *p<0.05.

4.2. Effect of circadian desynchrony on plasma biochemical parameters

Table 2. shows the plasma data of different biochemical parameters obtained using the previously detailed enzyme kits. Results are expressed as mean ± SEM in a concentration unit of mg/dL. Asterisks (*) represent statistically significant differences with respect to the control group and $ refers to the trend found with respect to the control.

Regarding TAG values, the chronodisrupted group treated with vehicle showed an increase of 44% in this value when is compared to the control group. This increase was slightly higher reaching almost a 70% when comparing the results of the chronodisrupted group treated with GSPE and the control. VLDL levels followed a similar pattern, being increased by 44% in the L11-VH group and by 62% in the L11-GSPE group. In phospholipids, significant differences could also be seen between the L11-GSPE group and the control. Also, it could be seen a trend between the L11-VH group and the control. These levels were 49.7% higher in the case of chronodisrupted group treated with vehicle compared to the control and 56% higher in the chronodisrupted group treated with GSPE compared to the control. Finally, a trend with increased values of glycerol could also be seen.

Table 2. Concentrations of the different plasma parameters (mg/dL) measured in samples of the three experimental groups. The results are expressed as the average ± SEM. Kruskal-Wallis non-parametric test and post-hoc Dunn's multiple comparison test was done. *p<0.005; **p<0.01; $: 0.05 < p < 0.06. The * symbol show the statistical differences compared to the control group and the $ symbol show the trend compared to the control group.

		Control	L11-VH	L11-GSPE
Glucose	**(mg/dL)**	107.52 ± 5.09	103.36 ± 2.82	101.03 ± 3.98
Cholesterol	**(mg/dL)**	123.23 ± 13.86	122.00 ± 15.26	132.14 ± 10.55
TAG	**(mg/dL)**	44.20 ± 2.22	63.67* ± 6.05	75.08** ± 7.58
HDL	**(mg/dL)**	20.75 ± 2.02	23.21 ± 1.63	21.07 ± 2.31
VLDL	**(mg/dL)**	8.84 ± 0.44	12.73* ± 1.21	14.40** ± 1.52
LDL	**(mg/dL)**	93.64 ± 13.46	86.06 ± 15.48	96.67 ± 11.58
NEFAs	**(mg/dL)**	29.25 ± 2.57	30.84 ± 2.13	32.20 ± 3.31
Phospholipids	**(mg/dL)**	75.37 ± 8.78	112.85$^{\$}$ ± 8.43	117.65* ± 10.07
Glycerol	**(mg/dL)**	10.05 ± 0.71	11.15$^{\$}$ ± 1.28	13.17 ± 3.70

To complete the information related to the lipid profile of the animals, different indices related with cardiovascular prognosis were determined. These results are collected in **Table 3**. In the same way that no significant differences were observed in plasma lipid levels, no significant differences were observed in any of these coefficients.

Table 3. Results of the different lipidic indices obtained. The results are expressed as the average ± SEM. Kruskal-Wallis non-parametric test was done.

	Control	L11-VH	L11-GSPE
Castelli Risk Index I	5.53 ± 0.76	5.47 ± 0.72	6.15 ± 2.06
Castelli Risk Index II	8.05 ± 0.74	9.51 ± 0.69	7.08 ± 1.89
Atherogenic Index (AIP)	2.35 ± 0.05	4.52 ± 0.06	3.35 ± 0.09
Atherogenic Coefficient (AC)	0.54 ± 0.76	0.36 ± 0.72	0.42 ± 2.06
Non-HDLc (NHC)	6.57 ± 13.53	8.96 ± 15.38	7.43 ± 11.02

4.3. Effect of circadian desynchrony on oxidative stress

To assess the oxidative status of the animals, it was firstly determined the hepatic MDA concentration in order to observe the degree of lipid peroxidation that was occurring. Secondly, we determined the hepatic activity of CAT to see the ability of the animals to reduce the oxidative stress generated.

The results of the hepatic concentration of MDA are shown in **Figure 18**. There were no significant differences between the groups.

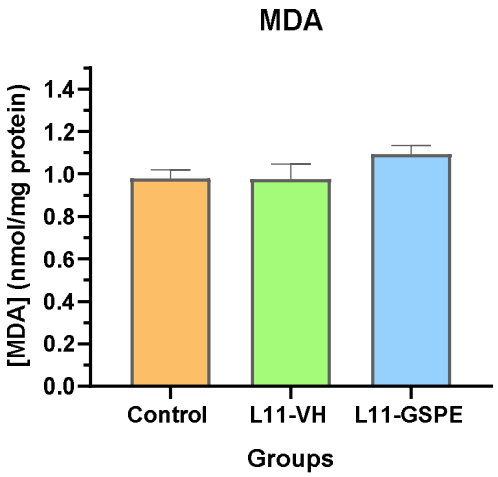


Figure 18. Representation of the hepatic MDA concentrations (nmol/mg protein). The results are expressed as the average ± SEM. Kruskal-Wallis non-parametric test was done.

Catalase activity is represented in **Figure 19**. It could be seen that, despite not being differences statistically significant between the three groups, the catalase activity in L11-VH group was lower if it was compared with both control and L11-GSPE groups.

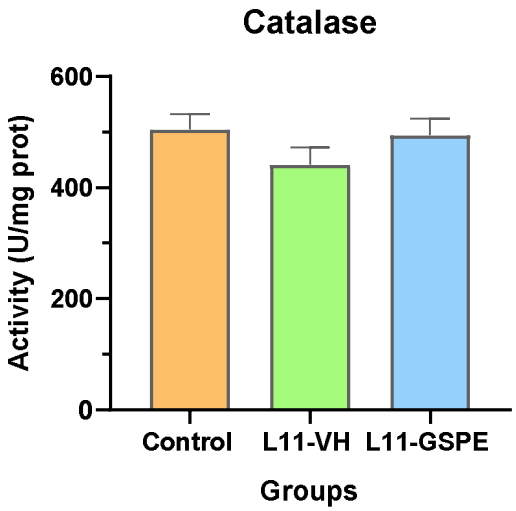


Figure 19. Representation of the hepatic CAT activity (U/mg protein). The results are expressed as the average ± SEM. Kruskal-Wallis non-parametric test was done.

4.4. Lipid profile analysis

With all the lipid parameters analysed, to evaluate if there were any lipidic profile characteristic that could differentiate the three groups, a multivariant statistical analysis was done with the Metaboanalyst v.5 platform. In this assay it was included the raw data of the weights of mesenteric, subcutaneous, perirenal, inguinal and epididymal fat depots, the AI, the plasmatic concentrations of cholesterol, TAG, HDL, LDL, VLDL, NEFAs, phospholipids and glycerol, and the hepatic concentration of MDA.

Figure 20. shows the Principal Component Analysis (PCA) obtained to see if the samples could be grouped with each other and classify them by their similarities. The red bubbles are the ones who represent the control group, the green ones are related to the chronodisrupted group treated with vehicle and, finally, the blues represent the chronodisrupted group treated with GSPE. There was a distinction in terms of the grouping of the samples between the red cluster and

the green, but it was not possible to clearly separate the blue cluster from the other two.

In this last blue cluster, the bubbles are more dispersed because the high variability of the samples. So, the blue cloud is overlapping the red and the green. It should be noticed that there are certain green and red points that are further from the rest, but it is only in a few of them in comparison to the blue group which is widely distributed.

In addition, this cluster aggrupation can represent correctly the 63.4% of the variability of the samples taking into account the result of the sensitivity of the three axes.

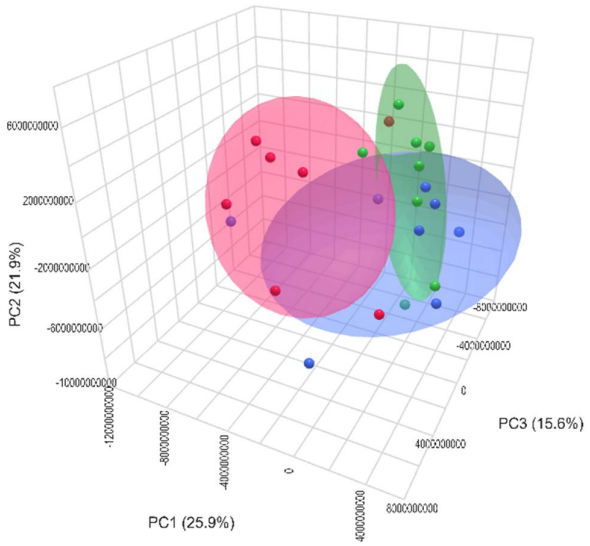


Figure 20. Principal Component Analysis for the lipid profile of the animals. In red are represented the samples corresponding to the control group; In green the L11-VH group; In blue L11-GSPE group.

In addition, the same data was represented in a heatmap form (**Figure 21.**), in order to visualize the data by measuring the magnitude of the phenomenon in two-dimensional colours.

Two main families could be observed with this grouping analysis. One was mainly constituted by the chronodisrupted animals treated with vehicle and, the other had all the animals from the control group but some of the chronodisrupted were also in it. In this representation it could be seen again the high variability that the chronodisrupted group treated with GSPE had because it could not be clustered differentially.

Also, it could be seen that the disrupted animals presented a remarkably high levels in the subcutaneous, inguinal and perirenal adipose tissue depots.

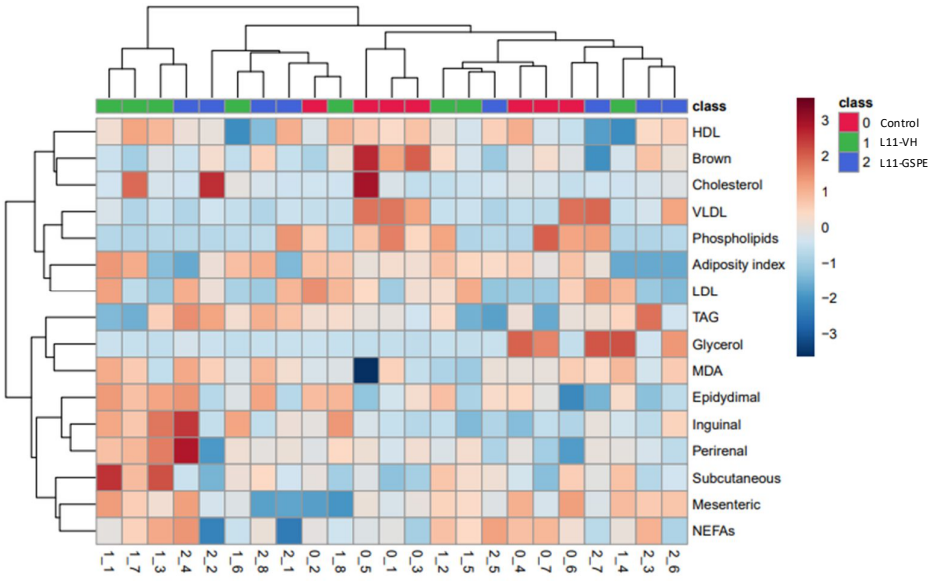


Figure 21. Heatmap representation for the lipid profile of the animals. Class red (0) represents the animals correspoinding to the control group; class green (1) the L11-VH; class blue (2) the L11-GSPE. The color scale means from minor (blue) to major (red) levels in the different paràmetres, passing through different shades in between, Columns are the samples introduced with the nomenclature “class_sample number”

5. DISCUSSION

More and more importance is being given to the chronodisruption factor as one of the main problems causing cardiometabolic diseases such as obesity and its comorbidities. For this reason, researchers are focusing all their efforts on finding cures or improvements for these pathologies under this approach. Exemplifying this fact, in obesity, which is considered the pandemic disease of the 21st century, there are different treatments that are not effective in the long term or that there is not enough information regarding the side effects. This is why the idea of looking for therapeutic strategies that restore circadian rhythms is being reinforced, such as the administration of natural products like GSPE (Liu et al., 2020).

In the results presented in this project, a clear affectation is observed in almost all parameters analysed due to the disruption of the circadian rhythm. Regarding the results obtained from the evaluation of the GSPE, it seems that in some parameters the dose of GSPE evaluated could re-establish the values presented by the animals in the control group, but it is not able to do so in all of them. In this regard, it is possible that the chronodisruption generated in the animals was so shocking that the dose administrated was insufficient to restore the damage caused in some pathways. In the bibliography consulted, trying to see which GSPE dose is more accurate to apply a high inconsistence was observed because it is highly dependent on different aspects such as the animal model. It should have taken into account that GSPE is a natural product which is produced by the secondary metabolism of the plants and the composition of this extract varies depending on the weather (stress environment conditions). Furthermore, maybe if we had done a longer treatment maintaining the dose it could be seen other results.

In the biometric measurements, a decrease in the weight of the rats with the disruption compared to the controls was observed since the first week. In the same way, this fact is reflected in the animals' food intake, observing a lower intake in the L11-VH group compared to the control. The differences in food intake between the period of light and darkness, showed that the disrupted animals had a completely opposite eating pattern to the animals belonging to the control group. In other words, during the dark phase, which represents the

animal's activity phase, the chronodisrupted groups had a lower intake compared to the control group. On the contrary, during the light phase, which is the period of inactivity or dormancy of the animals, they had a higher intake compared to the animals belonging to the control group. This fact could deeply affect the metabolic pathways and, as a consequence, the metabolism.

Regarding adipose tissue depots, significant differences are mainly observed in subcutaneous adipose tissue those corresponding to white adipose tissue (subcutaneous and perirenal depots). This fact is reflected in the AI being increased in L11-VH rats compared to controls and L11-GSPE. For the biochemical plasma analysis, there are statistically significant differences in the concentration of TAG, VLDL, phospholipids, and glycerol. Finally, no significant changes have been observed in the oxidative stress parameters in the animal model.

With these results obtained and contrasting them with the existing scientific evidence, trying to explain the reason why the L11-VH group had a lower body weight compared to the control group was quite challenging because in most articles it is considered that it should be related to an increase in weight. However, it is remarkable that our model is very particular and novel, and we only have the reference of one study (De Oliveira et al., 2019). So, the comparation of our results with other studies that were similar to our study was difficult. It is important to remind that the animals were healthy, as no obesogenic diet was applied, and the only external alteration was the disruption of the light/dark cycle.

In the bibliography consulted it is reflected how the timing of food intake can contribute to an increase in body weight in both animals and humans (Basolo et al., 2021). For example, in a study conducted with mice fed with a cafeteria diet, during their rest phase, they significantly increased their weight compared to those who were fed during the activity phase (Hernández-García et al., 2020). Similarly, mice fed with this same type of high-fat diet but with a restriction of intake, were found to be protected from obesity and hyperinsulinemia compared to animals that could consume similar caloric amounts of a high-fat diet in unrestricted time (Hernández-García et al., 2020). In another study, in which animals were fed with a standard diet, as we did, but in which animals were under restricted ingestion, they saw that the rats fed during the light phase showed an

accelerated increase in body weight promoting the appearance of obesity as well as changes in plasmatic glucose and TAG. Among other scientific evidence, it is shown that the metabolic effect of the calories consumed depends on the biological time at which they are consumed (Hernández-García et al., 2020). It seems that this is also related to the amount of melatonin secreted during the rest hours. This molecule is the main synchronizer of the body's peripheral clocks, and its release depends on the number of hours of sleep. It has been seen a relation between the melatonin concentration in the organisms and the misalignment between the different metabolic pathways as glucose and lipid metabolism compromising its homeostasis (Depner et al., 2014; Poggiogalle et al., 2018; Reiter et al., 2012). In our project, the mentioned point of view that melatonin can participate arranging the peripheral clocks cannot be confirmed because it has not been analysed yet. Interestingly, in the study of De Oliveira and colleagues, authors could not observe any body weight differences between the control and the disrupted group (De Oliveira et al., 2019).

If we compare the results obtained in the present project, regarding animals subjected to light/dark cycles of 12h/12h and those of 11h/11h treated with a vehicle, results similar to those presented above should have been observed since the food pattern of the chronodisrupted animals compared to the control animals was completely reversed. However, as explained, we observed a decrease in the body weight in the animals from the 11/11 group. This points to the complexity of this type of study, in which all these rhythmicity-dependent factors together could be affecting the pattern of the animal's body weight as well as their intake.

It has also been seen that the lifestyle in which the ingestion of food is promoted during the light phase can increase the probability of suffering from cancer due to the metabolic alterations (Hernández-García et al., 2020). This fact was seen in some of the chronodisrupted rats when they were sacrificed which presented tumours.

On the other hand, if the body weight of the control group is compared with the chronodisrupted group treated with GSPE, similarly to what happens with the L11-VH group, it presents lower values of the weight. Furthermore, despite not being significantly different between the L11-VH and L11-GSPE groups, those

treated with GSPE have even lower body weight values than those treated with vehicle. However, if the intake results are analysed, no significant differences are observed between the two chronodisrupted groups. In this case, there is scientific evidence that GSPE has the ability to reduce food intake due to different mechanisms, some of which still lack the necessary research to corroborate. In some studies, it has been seen that GSPE can inhibit certain digestive enzymes, the digestion of nutrients, and their absorption, reducing food intake (Yilmazer-Musa et al., 2012). However, other studies have shown that GSPE can inhibit b-glucosidase but, contrarily, improves the activity of amylases, lipases, and proteases which consequently improves the digestive capacity. This lack of consistency may be because different strains of rats are used. In addition, rats in their gastrointestinal tracts have few digestive enzymes and the variability between the results obtained can be very large (Liu et al., 2020). GSPE can act reducing the gastrointestinal motility by up to 60% in fasted animals or even up to 80% in animals that have just eaten (Penhoat et al., 2014). However, it has been shown that GSPE can modulate food intake by modifying the signal of glucagon-like protein-1 (GLP-1). GLP-1 is an incretin whose physiological function is to stimulate insulin production and decrease glucagon production, inhibiting appetite and intake, delaying gastric emptying, and increasing satiety. Therefore, it could be thought that, with the results obtained, despite the different existing evidence, the chronodisruption to which animals were subjected was so strong. So, the administration of GSPE was not sufficient to compensate for the effects of the alteration since there are no significant differences between the L11-VH and L11-GSPE groups. The differences that we can only observe are between both the disrupted groups and the control.

In relation to all this, it should be considered that the interpretation of the results related to chronodisruption, nutrition, and metabolism are very complicated to interpret since a large number of factors are involved. In addition, many studies are still needed to determine the different pathways that may be involved with all of this.

Summarizing the results obtained in adipose tissues and the adiposity index, it seems that, despite the body weight of the disrupted animals being lower than the control animals, they had a greater accumulation of fat in different adipose

tissues depots. This accumulation was especially observed in the white adipose tissue among perirenal and subcutaneous depots if we compare the L11-VH group with both the control and L11-GSPE groups. It has been described that, one of several mechanisms by which GSPE can reduce body fat content is based on its ability to act as a co-activator of the biliary acid nuclear receptor FXR (Liu et al., 2020). So, it could be possible that in this parameters GSPE is attempting to re-establish the parameters with respect to the control group as some scientific evidence had already shown.

It is also interesting to highlight the important anti-obesity role played by brown adipose tissue (BAT). Under healthy conditions, this tissue is characterized by consuming energy and generating heat. However, in pathological conditions, such as obesity, there is a significant decrease in temperature production due to mitochondrial dysfunction (Liu et al., 2020). In the results obtained, a decrease in the mass of brown adipose tissue is observed in the disrupted animals compared to the controls, although is not significant. On the contrary, the disrupted treated with GSPE have slightly higher values than those treated with vehicle, which may be an indication of the attempt to reverse the effect of the disruption in these animals. In relation to this, it has been seen that the administration of GSPE can promote mitochondrial respiration, improve mitochondrial function, and thus increase the heat production capacity of brown adipose tissue (Liu et al., 2020).

Certain biochemical parameters have also been shown to follow certain oscillatory patterns (Batotsyrenova et al., 2020). As it has been mentioned above, the significant increase in the concentration of plasma TAG in the two disrupted groups should be highlighted compared to the control. However, the levels of glycerol showed a tendency with an increased levels in the L11-VH group with respect to the control group, indicating that at the time of animal sacrifice, these could be using triglycerides as the main metabolic resource to obtain energy and therefore the release of glycerol in plasma. TAG are the form in which lipids are stored in adipose tissue. The fact that they are in high levels in plasma entails certain risks of suffering cerebrovascular accidents, heart attacks, and heart diseases due to the ability to contribute to the hardening of the arteries or the thickening of the arterial walls (arteriosclerosis). Due to this increase in plasma in TAG, consequently, an increase in the VLDL fraction is observed. These

lipoproteins are the way in which these TAG are transported through the plasma to other tissues so that they can be metabolized. At the hepatic level, the FXR receptor plays a very important role in controlling the homeostasis of TAG, cholesterol, bile acids, and glucose. The FXR receptor, in turn, can up-modulate the transcription factor SREBP1 which, when is activated, decreases the synthesis of fatty acids in the liver and increases the catabolism of TAG in plasma. When disruption occurs, all this homeostatic control performed by FXR and SREBP1c does not occur. The GSPE can re-establish this mechanism and, consequently, reduce the body's fat content (Liu et al., 2020). In the TAG concentration obtained, this re-establishment of the plasma TAG values cannot be observed. This probably is because it was also previously mentioned that the disruption was so strong that GSPE has not been able to improve the situation in some cases.

Phospholipids were also found in higher concentrations in the disrupted animals compared to the control. These molecules contain phosphates and are the main components of all biological lipid membranes, making these macromolecules essential components for cells. The determination of phospholipids is an important clinical indicator to diagnose alterations in the liver, mainly hyperbilirubinemia and also cardiac pathologies and diabetes.

With reference to the lipid profile of the animals, it should be noted that the proportion of HDL compared to LDL is quite low, a fact that can be observed in the calculation of the cardiovascular Castelli risk Index II. Another interesting index to analyse is the atherogenic index which, despite the difference not being statistically significant, is in the group of L11-VH animals where it has a value more than double that of the controls. This index reflects the esterification ratio of cholesterol by the enzyme Lecithin Cholesterol Acyl Transferase (LCAT) and the composition of subpopulations of lipoproteins that control the ratio. If the value of this index is less than 3.5 it is considered a minimal risk of suffering from a cardiovascular disease; if the values are between 3.51 and 4.5, they mean a moderate risk, and all those values higher than 4.51 are considered a maximum risk. In the results obtained, the control group would be at minimal risk of suffering from cardiovascular disease, in agreement with the state of health of the animals since they represent a model of a healthy individual. In the L11-VH group, they

are clearly at moderate risk of suffering from this type of disease. Finally, the L11-GSPE group would be at minimal risk of suffering from this type of disease, even though the value is almost at the limit of the established reference value.

In addition to this, resuming the results of biometric parameters related to blood pressure, it has been seen that a loss of rhythmicity is associated with a major risk on suffering cardiometabolic and cardiovascular diseases (Fabbian et al., 2013). Blood pressure is rhythmically regulated by many parameters that are also regulated in a circadian manner. So, in the results obtained it could be seen that the chronodisrupted groups from the fourth week the blood pressure started to destabilize and getting worse rhythmicity through the time and, therefore losing the dipper effect. With these results, we can see clearly the effect of chronodisruption, but GSPE had not the ability to compensate the disruption because the group treated had also lost the rhythmicity of the blood pressure. There is such a lack of scientific evidence related to blood pressure and circadian rhythms but in the study carried out by De Oliveira and colleagues showed also a loss of rhythmicity in this parameter and, interestingly, in the same way as us, there are not a hypertension situation. In other words, if it is compared the different mmHg of the SBP points the chronodisrupted rats did not show higher values compared to the control group.

In addition to the systolic blood pressure results, it has been seen in obese patients a relation between the blood pressure and the corporal distribution of fat. Some studies had related that major amounts of centrally fat located in the abdomen increase blood pressure although the peripheric accumulation of fat had a protective role in front of the blood pressure. Furthermore, also in obese patients, it had been seen that they had lost the circadian rhythmicity of blood pressure (Tałałaj et al., 2023). So, due to this evidence it should be possible that the chronodisruption affected the rhythm of blood pressure because of the difference fat accumulation between the control and chronodisrupted groups as it was discussed before.

Finally, regarding to the oxidative stress markers both MDA and catalase activity were not statistically significant different between the three groups. On the one hand, in MDA concentration we could not see any statistical differences between the groups. The responsible of the lipid peroxidation are ROS which interact with

the lipids bounded in the membrane cells generating this malondialdehyde intermediate so, we expected to have oxidative stress and consequently high levels of MDA. Contrary to our results, other studies with different sleep deprivation protocols had shown increased lipid oxidation in the liver (Villafuerte et al., 2015). It is true that oxidative stress molecules are highly unstable, they tend to react with the oxygen present in the environment to stabilize it so if the samples are not appropriately stored could interfere with the results. So, one limitation in this determination could be that we did not use an antioxidant in the sample storage. On the other hand, in CAT activity despite not being any statistical differences between the groups, it could be seen a decrease in the chronodisrupted group treated with vehicle in comparison to the control. In this case, we obtained similar results to De Oliveira and colleagues (De Oliveira et al., 2019). Also, similar results compared to other scientific evidence but employing a different chronodisrupted animal model have been described (Villafuerte et al., 2015). In contrast, the GSPE-treated chronodisrupted group in this case it seems to have the ability in increasing the activity of this enzyme and having the capacity to restore the health state of the animals.

Finally, the results showed in this bachelor final degree project are the initial ones and for understanding the final integration of what it was happening to the animals more investigation, in which is working, needs to be done.

6. CONCLUSIONS

According to the results obtained the principal conclusions responding to the different objectives set out in this project are:

- The forced circadian disruption suffered by the animals caused several changes in different markers.
  - Chronodisruption decreased the body weight, the food intake and altered the dietary pattern.
  - Some of the fat animal depots were increased due to the circadian disruption.
  - A loss of rhythmicity in systolic blood pressure was caused by the chronodisruption.
  - Related to the biochemical profile, the disruption caused an alteration in the lipidic profile, promoting a dyslipidaemia situation compared to the healthy animals.
  - The circadian alteration did not alter the oxidative stress markers evaluated.
- In general, the dose of Grape Seed Proanthocyanidins Extract used in the present study could not alleviate the alterations generated by the disruption.
  - Among the analysis carried out GSPE could reduce the amount of fat in the perirenal adipose tissue depot.

This work shows a new useful design for the investigation of the rhythm's misalignment, but further research is needed to evaluate the possibility of GSPE's application to treat the diseases related to the chronodisruption.

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