Příloha

Obesogens in Foods

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Review

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Abstract: Obesogens, as environmental endocrine-disrupting chemicals, are supposed to have had an impact on the prevalence of rising obesity around the world over the last forty years. These chemicals are probably able to contribute not only to the development of obesity and metabolic disturbances in individuals, but also in their progeny, having the capability to epigenetically reprogram genetically inherited set-up points for body weight and body composition control during critical periods of development, such as fetal, early life, and puberty. In individuals, they may act on myriads of neuro-endocrine–immune metabolic regulatory pathways, leading to pathophysiological consequences in adipogenesis, lipogenesis, lipolysis, immunity, the influencing of central appetite and energy expenditure regulations, changes in gut microbiota–intestine functioning, and many other processes. Evidence-based medical data have recently brought much more convincing data about associations of particular chemicals and the probability of the raised risk of developing obesity. Foods are the main source of obesogens. Some obesogens occur naturally in food, but most are environmental chemicals, entering food as a foreign substance, whether in the form of contaminants or additives, and they are used in a large amount in highly processed food. This review article contributes to a better overview of obesogens, their occurrence in foods, and their impact on the human organism.

Keywords: obesity; obesogens; food; adipose tissue; metabolic disruptors; systematic low-grade inflammation; metabolic syndrome

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1. Introduction

The rapid and significant increase in the prevalence of obesity worldwide over the last forty years is considered not to be attributed solely to genetic or life style risk factors, such as energy-dense and nutritionally poor diets, sedentary lifestyle, or aging. New evidence has shown that epigenetic, central regulatory pathways, and endocrine-disrupting changes that are associated with human exposure to man-made chemicals might also contribute to the obesity epidemic. So-called obesogens are xenobiotics directly or indirectly promoting adipogenesis and obesity in animals and humans, influencing individuals or their progeny. Many of these chemicals may also crossroad or modulate the effect of endogenous ligands of nuclear or non-nuclear transcription factors, participating in differentiation, metabolism, and the secretory function of adipocytes [1].

There are a number of examples in medicine that synthetically produced chemicals (drugs) may influence the development of adiposity. This usually adverse effect of pharmaceuticals is evidenced in glucocorticoids, estrogens, some antidiabetics (such as insulin, sulphonylureas, thiazolidinediones, glitazones), thyreostatics, dopaminergic blockers, beta sympathetic blockers, and, in some drugs, from the groups of tricyclic antidepressants, selective serotonin re-uptake inhibitors, atypical antipsychotic medicines, antiepileptics,

neuropeptides, and eutonics of the gastrointestinal tract [2–8]. However, not only medicines, but many compounds introduced in mega doses to the environment over the last decades by human production, were recognized to be able to act as obesogens. The main route of human exposure is dietary ingestion through contaminated food [9–11]. Also in the last 40 years, the dramatically developing food industry, using new technologies in the production of highly processed foods, can contribute to the development of obesity by changing the quality of food and the increased content of certain nutrients or additives [9].

2. Materials

We followed the current methodological guidelines for systematic reviews to identify, retrieve, and summarize the relevant epidemiological literature on the relation between obesogens and overweightness/obesity, Type 2 diabetes, metabolic syndrome, and atherosclerotic cardiovascular disease [12,13]. Each eligible paper was summarized with respect to the methods and results, with particular attention paid to the study design and exposure assessment. All articles were searched using Medline and Web of Science; we focused on the original articles and excluded doubled articles. We used the following search terms: "obesogens, metabolic disruptors, obesogens in food, food, additives, contaminants, obesity, adipose cells, adipose tissue, metabolic syndrome, systematic low-grade inflammation".

3. Obesity

Pre-obesity (overweight) and obesity are medical conditions marked by an abnormal and/or excessive accumulation of body fat that presents a risk to health (WHO 2019). According to the last definition, adopted by the European Commission in 2021, obesity is a chronic, relapsing disease, which in turn acts as a gateway to a range of other non-communicable diseases, such as diabetes, cardiovascular diseases, and cancer.

The obesity prevalence has risen exponentially in the world's population over the last 40 years. While in 1975, 6.4% of women and 3.2% of men were obese, the prevalence by 2014 roughly tripled to 14.9 and 10.8%, respectively. According to a prediction, every fifth adult will suffer from obesity in 2025. The global age-standardized mean body mass index (BMI) of children and adolescents aged 5–19 years has also been increased during the evaluated period from 1976 to 2016 in both genders, leading to virtually identical age-standardised mean BMIs for both genders [14].

The obesity pandemic has been probably brought about by dramatic changes in lifestyle during a relatively short period of human evolution. This maladaptation is the result of complex interactions between biological, behavioral, social, and environmental factors that are involved in the regulation of energy balance and fat stores.

In addition to increased mechanical load on the musculoskeletal system and cardiorespiratory load, obesity is a metabolic disease that is associated with dysfunctional white adipose tissue, affected by systematic low-grade inflammation. This leads to chronic systemic inflammation, ectopic fat accumulation in tissues and organs, a pro-coagulative state, endothelial dysfunction, and impaired carbohydrate, lipid, protein, and purine metabolism. It is linked to clinical conditions, such as hypertension, dyslipidemia, Type 2 diabetes mellitus, cardiovascular, and tumor diseases.

The reported estimates for the population-attributable risks of obesity have been shown to range from 5 to 15% for all-cause mortality, from 0.2 to 8% for all-cancer incidence, from 7 to 44% for cardiovascular disease incidence, and from 3 to 83% for Type 2 diabetes mellitus incidence [15]. Obesity is one of the leading causes of death and disability worldwide and is the fourth highest independent cause or premature mortality.

The histopathological unit of dysfunctional adipose tissue is characterized by adipocyte hypertrophy with infiltration of M1 macrophages, as well as impaired adipogenesis, angiogenesis, lipolysis, and de novo lipogenesis in adipose tissue.

4. Adipose Tissue

Adipose tissue is a complex, heterogeneous, and highly dynamic organ, executing the storage of energy and contributing to the control of energy metabolism of the whole organism. It consists of specific cells—maturate adipocytes that are differentiated under endocrine stimuli from their mesenchymal stem cell precursors during adipogenesis.

According to the morphology and function of the predominant maturated adipocytes three types of adipose depots are recognized in humans: the white (WAT), the brown (BAT), and the beige/brite/brown-like (BAT) adipose tissues. WAT contains adipocytes with a single large unilocular lipid droplet filling most of the cytoplasm and pushing the nucleus and organelles to the margins of the cells. BAT is characterized by smaller-sized adipocytes, with an abundance of smaller lipid droplets and many mitochondria. The third type—BAT—represents a combination of the attributes of previous two. Beige adipocytes are of middle size, have more lipid droplets and fewer mitochondria than BAT. It is supposed that WAT can be transformed into beige adipocytes under thermogenic stimuli [16].

Mature adipocytes are able under neuroendocrine control to store energy in lipid droplets in the form of triglycerides, and release it in the chemical (WAT) or thermal form (BAT) according to the body's requirements. The remaining cells are made up of stromal vascular fraction and belong to the immune, epithelial, vascular, and stromal cells. Besides storage and distribution of energy, adipose tissue contributes to the regulation of systemic energy metabolism by the secretion of adipokines that enables endocrine, paracrine, autocrine, and cross-talk communication with other organs. The physiological production of adipokines requires intact cellular machinery of mature adipocytes, in particular mitochondrial respiration and balance between lipogenesis and lipolysis. As hormones regulate the physiology of these systems, their action can be disrupted by chemicals in the environment that mimic or block normal endocrine functions [17].

Dysregulation of adipocytokines caused by obesity contributes to the pathogenesis of various metabolic and cardiovascular disorders [18].

5. Etiology of Obesity

Obesity and related disorders have become a public health issue [19,20]. As a multifactorial disorder, obesity cannot be linked specifically to one etiology, including genetics or environmental chemicals. While dietary restriction and increased exercise continue to be the most prescribed treatment, the obesity pandemic continues unabated and is increasing worldwide [21]. Despite the voluminous literature on obesogens and metabolism-disrupting chemicals, a series of workshops aimed at identifying the best evidence for the effects of these factors on obesity and diabetes have identified shortcomings in the available data that have prevented a complete and accurate analysis of their impact.

Obesity is most likely caused by (1) imbalance between energy intake and expenditure, resulting in energy surplus (e.g., by consumption of high-calorie diets); (2) genetic predisposition (40–70%), as well as hormonal, environmental, biological, psychological, and sociological factors; (3) lack of physical activity; (4) exposure to obesogens (endocrine disruptors or diabetogens) [22].

6. Obesogens

6.1. The Obesogen Hypothesises

The possible impact of obesogens, originally a highly controversial issue, has been supported by a growing body of evidence. Obesogens include xenobiotics that promote adipogenesis and obesity in animals and humans, such as several medicines or substances acting as endocrine disruptors [23]. Human activities have polluted water, soil, and foods. Obesogens are currently contained in many products for daily use, e.g., personal care products, cosmetics, cleaners, toys, kitchen utensils, plastic curtains and table cloths, soft furnishings, furniture, mattresses, and clothes.

Obesogens are chemicals that directly or indirectly increase fat accumulation and cause obesity [24]. The obesogenic hypothesis further suggests that obesogens can act directly at

the cellular level to increase the commitment or differentiation of adipocytes from stem cells by altering the number of adipocytes, increasing the retention of triglycerides within adipocytes, or modifying the rate of adipocyte proliferation when compared to cell death. Furthermore, obesogens can act indirectly as well by changing basal metabolic rate, shifting energy balance to favor calorie storage, and modulating food intake and metabolism via effects on the adipose tissue, brain, liver, pancreas, muscle, and the gastrointestinal tract [21,22].

To sum up, obesogens promote adipogenesis and fat accumulation, affect appetite control and satiety, and act as endocrine disruptors, possibly changing hormonal regulations [25].

The effects of obesogens can only become apparent later in life [26]. Previous studies have identified obesogens that have the potential to disrupt multiple metabolic signaling pathways in the developing organism, resulting in permanent changes to the adult's physiology. Prenatal or perinatal exposure to obesogenic endocrine-disrupting chemicals has been shown to predispose an organism to store more fat from early life [27].

This suggests that humans, who have been exposed to obesogenic chemicals during sensitive periods of development, might be pre-programmed to store increased amounts of fat, resulting in a lifelong struggle to maintain a healthy weight [24].

In this case, obesogens alone do not cause obesity in humans, but can work behind-the-scenes to promote weight gain, due to the developmental programming of adipose tissue regulation, poor diet, and metabolism [28].

In 2019, a study by Heindel and Blumberg provided strong evidence of the presence of estrogens acting as obesogens in humans. Since 2009 (the study of Newbold et al.), it has been known that the same holds for animals. In previous years, studies have identified transcriptomic and metabolomic changes of polychlorinated biphenyl-126 (PCB-126) in human hepatocytes, HepaRG, that imply the possibly detrimental role of environmental pollutants for the development of non-alcoholic fatty liver diease (NAFLD). These impacts might be precipitated by poor diet and/or a sedentary lifestyle [29]. Biological mechanisms acting in the development of hepatic steatosis are divided into four categories: increased fatty acid uptake, decreased lipid efflux, increased fatty-acid synthesis, and impairment of the oxidative metabolism of these substances [30]. The further elucidation of impaired hepatic lipid metabolism is needed [31]. Animal studies have clarified the impact of obesogens on the etiology of obesity. Currently, they focus on human lipid metabolism. Tissue culture studies are being carried out predominantly on 3T3-L1, derived from mouse cells. The main aims of 3T3-L1 studies are the clarification of the obesogenic potential of xenobiotics and their metabolites, as well as the assessment of their impact on adipose differentiation. Xenobiotics may increase the number of differentiated 3T3-L1 pre-adipocytes and enhance their capacity for droplet storage. The mechanism behind is most probably the up-regulation of transcription factors CCAAT/enhancer-binding protein α (C/EBP α) and peroxisome proliferator-activated receptor Ɣ (PPARƔ). These are associated with significantly higher expression of fatty-acid-binding protein 4 adipokine [32].

PPAR is a ligand-activated transcription factor, which is responsible for the growth and development of adipose tissue and that acts as the receptor for antidiabetic drugs such as rosiglitazone [33,34]. Neither the mechanism nor the modification of the key cellular processes lying between induction of the receptor and onset of the disease have been described [35,36].

The most common contaminants that are considered to be potential obesogens include estrogens, such as diethylstilboestrol and genistein; organotins, e.g., tributyltin; fluoro actanoates; bisphenol A; diethylhexyl phthalate. These chemicals directly alter endocrine function and metabolic organs that control lipid homeostasis (e.g., the liver), suggesting that exposure might be a risk factor for the development of NAFLD [22,23,37–39].

In 2015, the Parma consensus broadened the definition of obesogens to include endocrine disrupting chemicals that affect other obesity-related metabolic conditions that drive metabolic syndrome, such as insulin resistance, hypertension, dyslipidemia, and

hyperglycemia [17]. This class of endocrine-disrupting chemicals was denoted as being metabolism-disrupting chemicals [40].

6.2. Overview of Obesogens

Presence in foods

1. ***Naturally occurring obesogens***
   - Fructose
   - Genistein
2. ***Xenobiotics***
   - 2.1 **Contaminants**
     - Pharmaceuticals
       - Diethylstilbesterol
       - Estradiol
       - Rosiglitazone
     - Organic Pollutants (OP's)
       - **Industrial Chemicals**
         - Bisphenol A (BPA)
         - Organotins
         - Perfluorooctanoic Acid (PFOA)
         - Phthalates
         - Polybrominated Diphenyl Ethers (PBDEs)
         - Polychlorinated Biphenyl Ethers (PCBs)
       - **Organophosphate Pesticides**
         - Chlorpyrifos
         - Diazinon
       - **Organophosphate Pesticides**
         - Dichlordifenyltrichloretan (DDT),
         - Dichlordifenyltrichloretan (DDT),
       - **Other Environmental Pollutants**
         - Benzo[a]pyrene
         - Fine Particulate Matter ($PM_{2.5}$)
         - Triclosan
   - 2.2. **Additives**

1. ***Naturally occurring obesogens***

Fructose

Fructose, a monosaccharide present in fruits and honey, promotes the development of obesity easier than glucose. Its overconsumption contributes to the increasing prevalence of obesity, insulin resistance, and metabolic as well as cardiovascular diseases [41]. Fructose is capable to affect the intestinal microflora with increased intestinal permeability [42]. Fructose-2,6-bisphosphate derived from fructose-6-phosphate has been identified as one of the signaling metabolites responsible for glucose-induced recruitment of carbohydrate response element binding protein (ChREBP) to its target genes. ChREBP promotes de novo lipogenesis in liver and adipose tissue [43,44].

Due to the different metabolism and high lipogenic potential by fructose when compared to glucose, fructose ingestion precipitates the accumulation of excessive fat in the liver and results in weight gain and abdominal obesity [45].

Recently, fructose has become overabundant in the food industry, especially in the case of non-alcoholic sweetened beverages and sweets.

Genistein (in soy)

Phytoestrogens, contained in various foods and food supplements, in particular soy products, are another prominent class of chemicals. Genistein and daidzein are two of the most abundant phytoestrogens in the human diet. For its estrogenic activity, genistein has been proposed to have a role in preserving good health by regulating lipid and carbohydrate homeostasis [46]. Genistein is also used as a supplement for menopausal

woman. However, a recent study showed that only at high doses did genistein indeed inhibit adipose deposition, but, at low doses similar to that found in Western and Eastern diets, in soy milk or in food supplements containing soy, it surprisingly induced adipose tissue deposition, especially in males. Further, this increase in adipose tissue deposition by genistein was correlated with mild peripheral insulin resistance. Interestingly, genistein did not significantly affect food consumption [47] suggesting an abnormal programming of factors involved in weight homeostasis [48].

2. *Xenobiotics*

Xenobiotics are not natural compounds found in foods and are not used as separate foods. The presence of xenobiotics in foods, according to the dose, can be harmful to humans.

Substances that are not naturally occurring compounds of foods are called foreign substances. Foreign substances or xenobiotics are classified either as contaminants or as additives.

2.1. Contaminants

Substances contaminating foods unintentionally, not posing a risk in usual concentrations but being potentially harmful at higher doses, are called contaminants. They can contaminate food. Contamination may occur at each step of the production chain.

The most common causes of contamination are: the use of veterinary drugs, contaminated soil from environmental pollution, persistent organic pollutants for agricultural purposes, sanitary materials, radioactive contaminants, traffic pollutants, and contamination from packaging materials.

Substances contained in packaging materials, such as phthalic acid, are used as a softener.

The primary contaminants of high concentration include toxic metals, above all being lead, mercury, cadmium, and inorganic chemicals, e.g., nitrous and nitric oxide.

2.1.1. Pharmaceuticals

Some pharmaceuticals used in veterinary medicine and in animal production may act as possible obesogens in humans:

Diethylstilbesterol (DES)

This is an estrogen that was prescribed to millions of women from 1940–1971 to prevent abortion in the first trimester of pregnancy. The prescription has been suspended due to adverse side effects, but the drug is still being used to enhance fertility in livestock and, therefore, enters the food chain. DES may have acted an obesogen in the human population [49].

2.1.2. Organic pollutants (OPs)

These toxic and cancerogenic chemicals are very resistant to degradation and many of the products of their decomposition are toxic as well. The greatest risk stems from their ability to accumulate in the food chain. The main source of OPs are animal foods (meat, fatty fish, dairy products, and eggs).

2.1.2.1. Industrial chemicals

Bisphenol A (BPA)

BPA is one of the highest-volume chemicals used in commerce. Its omnipresence in polycarbonate plastics, epoxy resins (automobile parts, safety protective equipment, food and water containers, baby bottles, or the protective lining inside metal food cans, dental fillings, etc.), and thermopaper contributes to continuous human exposure [50,51]. Dietary ingestion is suspected to be the main route for human exposure, although dermal exposure can also occur from skin contact with thermal paper. BPA has been detected at measurable concentrations in the urine samples of almost all persons tested worldwide. In addition, BPA has been detected in placental and amniotic fluids and human breast milk (Blumberg 2021).

BPA is an endocrine disruptor exhibiting estrogen-like activity that is able to affect the regulation of leptin and insulin production, and thus acts as an agonist and antagonist of PPARγ [52].

Many studies clearly support the enhancement of adipogenesis, dysregulation of adipocytes and glucose, and the inflammatory changes of adipose tissue resulting from BPA, resulting in obesity [53,54].

A systematic review with a meta-analysis of the epidemiological evidence, given by Wu et al., has revealed a positive correlation between the level of BPA and obesity risk. A dose–response analysis revealed that a 1 ng/mL increase in BPA increased the risk of obesity by 11%. There were similar results for different types of obesity, gender, and age [55].

Due to its adverse effects on human health, the European food safety authority (EFSA) has determined the tolerable daily intake of BPA (4 µg per kilogram of body weight per day) [56]. Today, there is a growing tendency to replace BPA with its analogues. This is based on legal limits for BPA in basic goods. In 2019, a longitudinal cohort study revealed a significant association of bisphenol S (BPS) and bisphenol F (BPF) with obesity in children aged 6 to 19 when compared to total bisphenol and BPA. The replacement of BPA with other bisphenols therefore might not be efficient [57].

Organotins (OTs)

These are chemicals widely used as pesticides, disinfectants, and biocides in paints. OTs are harmful to endocrine glands and can interfere with neuroendocrine control, hormone synthesis and/or the biological availability or activity of target receptors. They impair metabolism either centrally (lateral hypothalamus) or peripherally (adipose tissue) and result in obesity. Besides their obesogenic effects, OTs affect reproductive organs [58,59]. Due to their physical and chemical properties, OTs easily enter food chains and produce tributyltin (TBT) and triphenyltin, which are both severely toxic [60,61].

The most common OTs include TBT. Pilot studies have shown a positive correlation between placental TBT concentration and weight gain in infants [62,63]. Humans are exposed to TBT in seafood, foods treated with agricultural fungicides and miticides, industrial waters, textile material, polyvinyl chloride stabilized with TBT, or food packaging. Indirectly, house dust, which contains significant amounts of TBT, can be the source of contamination [64,65].

Perfluorooctanoic Acid (PFOA)

This is a group of synthetic chemicals that is used for their high resistance and stability. They are intermediates of Teflon production, and are commonly found in the environment. The route of exposure is mostly the digestive tract.[4] Evidence supports the obesogenic effect of PFOA, though its biological mechanism needs further clarification. A study by Lia et al. (2020) demonstrated that the obesogenic effect of PFOA was the result of a combination of many enzymatic pathways with insulin signaling [66]. In 2022, a study supporting previous findings suggested that PFOA might act as a developmental obesogen, transmitted vertically via the placenta [67].

Phthalates

Phthalates or phthalic esters form a group of chemicals that are used as softeners for plastics, additives for cosmetics, insecticides, or as adhesives. They can be detected in breast milk and enter foods from packaging materials, including package water and spirits, but they are particularly present in fatty foods, because they are lipophilic. In addition, toys containing phthalates can enter the organism when placed into the mouth [68]. Phthalates are one of the most studied metabolic disruptors. Several observational studies suggested that phthalates could be determinant in the pathogenesis of obesity [69]. Phthalates act as thyroid hormone agonists as well as androgen agonists. Thus, they can affect adipogenesis, fat accumulation, and insulin resistance by interfering with PPAR activation [70]. A recent study from 2020 suggests an association of child growth with prenatal exposure to phthalates, especially those of low molecular weight.[71] Furthermore, in the case of chronic exposure to low doses of phthalates, adverse effects (spermiotoxic, embryotoxic,

and teratogenic) on the reproductive system were observed, as well as hepatotoxicity and nephrotoxicity [68,72].

Polybrominated Diphenyl Ethers (PBDEs)

PBDEs were used as flame retardants in plastics, electronics, vehicles, households, furniture, textile material, and building materials. Several studies showed an association between PBDEs and foods, as they were detected in butter, fish, and other products high in animal fat [73]. Despite a production ban due to adverse effects on human health, the use of reserves is still allowed. One of the most common PBDEs is PBDE 99, which can be detected the most in adipose tissue, especially white adipose tissue [74,75]. An epidemiological study demonstrated a positive correlation between early exposition to PBDEs and increased adiposity at the age of 8 years [76]. This has been confirmed at the cellular level with PBDE 99 and the adipocyte lineage of C3H10T1/2 [77]. These studies support the obesogenic effect of PBDEs. In addition to a pro-adipogenic effect in cell cultures 3T3-L1, they increase fat accumulation, as well as C/EBA α and PPARγ expression, in the course of differentiation [78].

Polychlorinated Biphenyl Ethers (PCBs)

This is a group of fat-soluble chemicals that differ from each other in the number and position of chlorine bound to the biphenyl. There are 2010 PCB congeners. Due to their industrial use in paints, varnishes, plastics, pesticides, and coolants, they have entered the environment and foods. Long-term consumption of food containing high amounts of PCBs might be hazardous [26,79]. These are especially milk, fish, and animal foods. Animal products are contaminated via agricultural premises that have not removed paint used before 1986, before the use of PCBs was banned [80]. Animal studies suggest that PCBs promote the differentiation of adipocytes and PPAR expression, resulting in weight gains in offspring [25]. The obesogenic effect of PCBs is discussed in many studies [81]. Valvi et al. demonstrated that PCB concentration in cord blood was associated with BMI and overweightness in children at the age of 5, 6.5, and 7 years, showing a more profound effect in girls [82,83].

2.1.2.2. Organophosphate Pesticides (OPPs)

More than 100 various organophosphates have been described. OPPs pertain to the most commonly used pesticides worldwide and their use in agricultural premises has rapidly increased. The World Health Organization (WHO) has designated OPPs as being extremely hazardous [84,85].

Chlorpyrifos (CPF)

This is an organophosphate pesticide widely used in agriculture and, therefore, has entered the environment. Today, studies on mice 3T3-L1 models are being carried out and a study by Blanco et al. suggests that CPF and its metabolite 3,5,6-trichlorpyridinol (TCP) affect metabolism during adipogenesis, by increasing the number of differentiated 3T3-L1 adipocytes and the capacity for storage of lipid droplets. This process is linked to an up-regulation of the transcription factors CCAAT/enhancer binding protein α (C/EBP α) and PPARγ, which is accompanied by a significantly higher expression of fatty acid-binding protein 4 (FABP4) adipokine [86,87].

Diazinon

This pesticide and nematocide was widely used in agriculture and commonly detected in the human population. Residues of diazinon were also detected in ground water and drinking spring water [88]. Via inhibition of acetylcholinesterase, diazinon elicits neurotoxicity. Its pro-adipogenic effect has been shown in a study on mice 3T3-L1, where the accumulation of lipid droplets and the activation of proadipogenic signaling pathways were related to the concentration of diazinon. Diazinon significantly induced the protein expression of the transcription factors CCAAT-enhancer-binding proteins α (C/EBP α) and PPARγ, as well as their downstream proteins, fatty-acid synthase (FASN), acetyl CoA carboxylase, lipoprotein lipase, adiponectin, perilipin, and fatty-acid binding protein 4 (FABP4) [89].

2.1.2.3. Organochlorinated Pesticides (OCPs)

Dichlordifenyltrichloretan (DDT),
Dichlorenthylendichlordiphenyldichlorethylen (DDE)

The insecticide DDT was used on a large scale from 1939 against mosquitos *Anopheles funestus*, a vector of malaria. After its toxicity had been demonstrated, DDT was banned for use. There are exceptions to this rule, especially in developing countries that are fighting malaria. Problems with DDT and its products of degradation have continued until today because of its continuous presence in the environment. Due to its persistence, DDT has entered the food chain and has often been detected in animal adipose tissue and water. The largest part of DDT and its metabolites enter the human organism via the consumption of meat, dairy products, and fish. Leafy vegetables are usually richer in DDT when compared to other kinds of vegetables. Breast-feeding is another important form of human exposure. A growing amount of epidemiological evidence, in both in vivo and in vitro studies, have associated persistent organic pollutants, such as DDT and the DDT metabolite p,p'-DDE, with obesity [90–93]. Acute exposure causes harm to the central nervous system, while chronic exposure can result in liver cancer, disruption of endocrine control, harm of the fetus and fertility, and increased risk of Type 2 diabetes. DDT and especially its metabolite DDE may pose a risk of developing obesity in later life [94]. Rodents exposed to DDT during prenatal life have been found to have decreased energy expenditure along with glucose intolerance, dyslipidemia, and hyperinsulinemia [95].

2.1.2.4. Other Environmental Pollutants

Benzo(a)pyrene

This is a polycyclic aromatic carbohydrate present in smoke and a proven carcinogenic chemical that is produced in the course of burning, grilling, or smoking foods [96]. Its anti-adipogenic effect via the aryl hydrocarbon receptor has been demonstrated on cell cultures of human preadipocytes [97,98].

Triclosan

This is a commonly used antibacterial agent, present in oral care waters, toothpastes, toothbrushes, antibacterial soap, washing powder, and kitchen breadboards. Exposure to triclosan and triclocarban has been linked to an elevated risk of child obesity [99]. Animal studies show a correlation between high levels of triclosan and estrogens, androgens, and thyroid hormones. Human stem cell culture models have demonstrated an anti-adipogenic effect, including a lower production of adiponectin and lipoprotein lipase (i.e., markers of cellular fat), which is correlated to the concentration of the chemical [100].

Fine Particulate Matter ($PM_{2.5}$)

In previous decades, particulate pollution has become a growing health issue worldwide, especially in the northern and north-western regions of China [101]. Motor transportation has considerably contributed to the concentration of PM in urban areas, as well as biomass, other waste, or industrial burning or road dust [102]. Several studies have shown an association between air pollution and the risk of obesity, predominantly in the male population [103].

2.2. Additives

Commonly used additives have been linked to obesity. These substances include the emulsifiers carboxymethylcellulose and P-80, the surfactants DOSS and Span-80, the preserving agent 3-tert-butyl 4-hydroxyanisol (3-BHA), artificial sweeteners, and the flavor enhancer monosodium glutamate (MSG) [104–106]. More than 350 additives have been approved in the European Union and we have focused only on examples of the most discussed.

Monosodium glutamate

This is a chemical eliciting the secretion of glucagon-like peptide-1, a hormone controlling appetite and satiety, and/or antagonization of the androgen receptor [107,108]. That MSG contributes to the early onset of obesity has been demonstrated in animal studies. Mice administered with MSG postnatally showed a significantly increased proportion of

fat in both sexes. MSG administered postnatally to mice acted as a neurotoxic agent on the hypothalamic arcuate nucleus, leading to obesity [109,110].

These findings represent a promising outlook for future research, as they draw attention to the consequences of a highly processed diet.

Carrageenan

This is a hydrocolloid substance, commonly present in chocolate milk and ice cream, that is able to impair glucose tolerance, increase insulin resistance, and inhibit insulin signaling in in vivo mouse liver cells and human HepG2 cells. A study on mice from 2021 showed a significant change in gene expression related to lipid metabolism, especially in the decreased gene levels of adipocytokines, lipogenesis, lipid absorption, and transport, and the increased genes for adipolysis and oxidation after carrageenan exposure [111,112].

Antioxidants

Foods often contain antioxidants, such as the preserving agent natrium sulphite, natrium benzoate, natural coloring agents, and curcumin. Oxidative stress, caused by the consumption of additive artificial antioxidants in foods at a younger age, has been associated with the development of adiposity in later life.

Lower leptin secretion in mouse adipocytes 3T3-L1 after incubation with LPS mimicked inflammation in obesity, i.e., consuming antioxidant additives might lead to lower leptin secretion and contribute to the obesogenic environment [113,114].

All mentioned obesogens have been shown in Table 1.

Table 1. Obesogens and their effect on human health.

		Obesogens	Obesogenic Effect	Health Impact
Naturally occurring obesogens		Fructose	Different fructose metabolism and high lipogenic potential = excessive fat storage in the liver, weight gain of visceral adipose tissue. In fetal, neonatal and infant development, high exposure to fructose as an obesogen can affect lifelong neuroendocrine function, appetite control, eating behavior, adipogenesis, fat distribution [45].	obesity, insulin resistance, metabolic and cardiovascular diseases
		Genistein	At high (pharmacological) doses it inhibits adipose tissue deposition, but at low doses (normal concentration in soy) it induces adipose tissue deposition, especially in men. The genistein regulate estrogen and progesterone receptors [47].	Obesity, mild peripheral insulin resistance
		Obesogens	**Obesogenic Effect**	**Health Impact**
Xenobiotics	Pharmaceuticals	Diethylstilbesterol	Endocrine disruptor with abnormal programming of various differentiating estrogen-target tissues [49].	Potential obesogen
		Estradiol	Estradiol in combination with a diet rich in fats and sugars causes variability in estrogen-induced gene expression in the dorsal raphe [7].	Potential obesogen
		Rosiglitazone	Rosiglitazone reduces hyperlipidemia and hyperglycemia, improves insulin sensitivity and decreases serum lipids, but does increase adipogenesis and lipid accumulation in tissues including liver triglyceride accumulation and hepatic steatosis [6].	Potential obesogen

Table 1. *Cont.*

Industrial chemicals	Bisphenol A (BPA)	Endocrine disruptor, it is able to affect regulation of leptin and insulin secretion (PPARγ agonist and antagonist) [50].	Supports adipogenesis, dysregulation of adipocytes and glucose, inflammation of adipose tissue → obesity
	Organotins (OTs)	OTs can damage the endocrine glands, interfering with neurohumoral control of endocrine function involves changes in the mechanism of adipose tissue [58].	Predisposition to obesity, metabolic disorders, and effects on reproductive organs
	Perfluorooctanoic Acid (PFOA)	PFOA can cause aberrant lipid metabolism in male offspring, insulin resistance, non-alcoholic fatty liver disease, with influencing PPARγ signaling pathway [66,115].	Obesity, hepatic inflammation, disorders of lipid metabolism, disruption of gut barrier integrity in male offspring
	Phthalates (di-(2-ethylhexyl) phthalate (***DEHP***), di-butyl phthalate (***DBP***), benzylbutyl phthalate (***BBP***) and possibly also di-isononyl phthalate (***DINP***), di-isodecyl phthalate (***DIDP***) and di(n-octyl)-phthalate (***DNOP***)	Phthalates can cause insulin resistance, increase endoplasmic reticulum expression, disruption of glucocorticoid signaling in mesangial cells and preadipocytes [70].	Predisposition to obesity and metabolic diseases can influence metabolic regulation by disrupting the homeostasis of thyroid hormones
	Polybrominated Diphenyl Ethers (PBDEs)	PBDEs are insulin disruptors, isoproterenol stimulates the metabolism of adipocytes [116–118].	Predisposition to obesity, insulin resistance in obese individuals
	Polychlorinated Biphenyl Ethers (PCBs)	PCBs are lipophilic toxicants into adipocytes. In particular, the degree of halogenation or the number and position of chlorine substituents on PCBs affects their uptake and accumulation in adipocytes [119].	Predisposition to obesity, metabolic disorders (disruption of adipose tissue function)
Organophosphate pesticides	Chlorpyrifos	Chlorpyrifos can cause an increasing number of differentiate 3T3-L1 adipocytes and the capacity for storage of lipid droplets due to up-regulation of transcription factors CCAAT/enhancer binding protein α (C/EBP α) and PPARγ, which is accompanied by significantly higher expression of fatty acid-binding protein 4 (FABP4) adipokin [86].	Metabolic disorders, obesity
	Diazinon	Via inhibition of acetylcholinesterase, diazinon elicits neurotoxicity, significantly induces protein expression of transcription factors CCAAT-enhancer-binding proteins α (C/EBP α) and PPARγ as well as their downstream proteins, fatty-acid synthase (FASN), acetyl CoA carboxylase, lipoprotein lipase, adiponectin, perilipin, and fatty-acid binding protein 4 [89].	Obesity, neurotoxicity

Table 1. *Cont.*

	Organochlorinated pesticides	Dichlordifenyltrichloretan (DDT), Dichlorenthylen-dichlordiphenyldichlorethylen (DDE)	DDT, DDE can cause disruption of endocrine control, glucose intolerance, dyslipidemia, and hyperinsulinemia [120].	Acute exposure causes harm to the central nervous system, while chronic exposure can result in liver cancer, obesity, harm for the fetus and fertility and increased risk of Type 2 diabetes
	Other environmental pollutants	Benzo[a]pyrene	It can be originator cytotoxicity and expression of inflammation markers [121].	Predisposition to obesity, non-alcoholic fatty acid disease, asthma, hepatic steatosis
		Fine Particulate Matter ($PM_{2.5}$)	$PM_{2.5}$ may cause adipose tissue inflammation [103].	Risk of obesity, predominantly in the male population
		Triclosan	Animal studies show a correlation between high levels of triclosan and estrogens, androgens and thyroid hormones [100].	Risk of obesity
Additives		Monosodium glutamate	It induces the secretion of glucagon-like peptide-1, a hormone controlling appetite and satiety, and/or antagonization of the androgen receptor, act as a neurotoxic agent on hypothalamic arcuate nucleus and lead to obesity [107,108].	Obesity
		Carrageenan	Is able to affect glucose tolerance, increase insulin resistance and inhibit insulin signaling in in vivo mouse liver cells and human HepG2 cells, promote significant changes in gene expression related to metabolism and lowering of adipokine genes, as well as lipogenesis, absorption, and transport of lipids. Adipolysis and oxidation increase [111,112].	Predisposition to obesity
		Antioxidants	Consuming antioxidant additives might lead to lower leptin secretion and contribute to the obesogenic environment [113,114].	Predisposition to obesity

7. Obesogen Elimination Method

The common occurrence of obesogens with which humans are in regular contact should be limited. Because humans are already heavily exposed to environmental obesogens in the form of plastics, pesticides, herbicides, industrial products, and personal care products, compounds intentionally added to foods, such as certain artificial sweeteners, phytoestrogens, preservatives, added sugars (e.g., corn syrup with a high fructose content) deserve special attention. Furthermore, it has already been proven that many obesogens are found in animals and their products, which we then use as food [106–111].

Another way to eliminate obesogens is to consume organic products that are not treated with pesticides, fungicides, and other sources of obesogens. Fruits and vegetables

are commonly treated with fungicides that have been identified as obesogens, such as glyphosate used on corn, wheat, and rice [122–124].

Food processors also deal with the issue of obesogens. Studies are being sought looking for methods of eliminating obesogens from food. For example, Rezaei et al. published a study in 2021 to remove the pesticide diazinon from apple juice and found a successful solution using fermentation with the cultivated bacterium *Lactobacillus acidophilus*, where the product is stored in the cold for 28 days after fermentation and the diazinon is completely removed [125].

8. Conclusions

The study of obesogenic compounds in food is still in its early phase, and people are constantly exposed to obesogens, either directly from food or contaminated food. Regarding the objectives of food industry technologies, i.e., the extension of expiration dates, cost reduction, the best attainable palatability, optimization of production effectiveness, and food safety in terms of the absence of pathogens, over 4000 new substances have entered into foods. In most of the new substances, their impact on overall metabolic homeostasis remain unknown. Many of these xenobiotics may, as obesogens, cause epigenetic, central regulatory pathway, and endocrine-disrupting changes, directly or indirectly promoting adipogenesis and obesity in humans, influencing individuals or their progeny. Many of them may also crossroad or modulate the effect of endogenous ligands of nuclear or non-nuclear transcription factors, participating in differentiation, metabolism, and the secretory function of adipocytes. Metabolism-disrupting chemicals may affect other obesity-related metabolic conditions that drive metabolic syndrome, such as insulin resistance, hypertension, dyslipidemia, and hyperglycemia.

Confirmation of the effect of obesogens at the current exposure concentrations for the general population still requires a larger number of scientific studies to support better management of these chemicals in our environment, and to decrease human exposure.

The biological effects of main obesogenic candidates can be correctly analyzed to obtain data to advocate for the requirement to revise their regulation. Efforts should be made to better regulate the production of these obesogens and metabolic disruptors, their use and, therefore, decrease environmental and food contamination. In addition, new approaches and ways to minimize their obesogenic, and especially metabolic-disrupting, potential in humans should be under investigation, which could help to develop an efficient strategy to reverse the increasing trend of the obesity pandemic.

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Mitochondrial Respiration of Adipocytes Differentiating From Human Mesenchymal Stem Cells Derived From Adipose Tissue

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Chronic DDE Exposure Modifies Mitochondrial Respiration during Differentiation of Human Adipose-Derived Mesenchymal Stem Cells into Mature Adipocytes

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Abstract: The contribution of environmental pollutants to the obesity pandemic is still not yet fully recognized. Elucidating possible cellular and molecular mechanisms of their effects is of high importance. Our study aimed to evaluate the effect of chronic, 21-day-long, 2,2-bis (4-chlorophenyl)-1,1-dichlorethylenedichlorodiphenyldichloroethylene (p,p′-DDE) exposure of human adipose-derived mesenchymal stem cells committed to adipogenesis on mitochondrial oxygen consumption on days 4, 10, and 21. In addition, the mitochondrial membrane potential (MMP), the quality of the mitochondrial network, and lipid accumulation in maturing cells were evaluated. Compared to control differentiating adipocytes, exposure to p,p′-DDE at 1 µM concentration significantly increased basal (routine) mitochondrial respiration, ATP-linked oxygen consumption and MMP of intact cells on day 21 of adipogenesis. In contrast, higher pollutant concentration seemed to slow down the gradual increase in ATP-linked oxygen consumption typical for normal adipogenesis. Organochlorine p,p′-DDE did not alter citrate synthase activity. In conclusion, in vitro 1 µM p,p′-DDE corresponding to human exposure is able to increase the mitochondrial respiration per individual mitochondrion at the end of adipocyte maturation. Our data reveal that long-lasting exposure to p,p′-DDE could interfere with the metabolic programming of mature adipocytes.

Keywords: human adipose-derived mesenchymal stem cells; adipogenesis; p,p′-DDE; mitochondrial respiration

1. Introduction

Adipose tissue is a complex heterogeneous and highly dynamic organ performing many functions. It contributes to the control of energy metabolism of the whole organism. The specific function of adipose tissue is to provide mature adipocytes, i.e., cells that are able to store energy in lipid droplets in the form of triglycerides and release it in the chemical or thermal form according to the body requirements [1]. Adipogenesis, the process during which the mature adipocytes differentiate from their precursors, mesenchymal stem cells, is necessary for the dynamic renewal and optimal function of adipose tissue. It is supposed

that the increase in adipocyte number is triggered by signaling factors that induce the commitment of pluripotent mesenchymal stem cells residing in the vascular stroma to the adipocyte lineage. When committed, preadipocytes are subjected to mitotic clonal expansion undergoing two or three cell divisions and then they gradually acquire typical adipocyte metabolic and morphological characteristic in the process of differentiation [2]. In the course of adipogenesis, mitochondrial oxygen consumption progressively increases. During the first days of adipogenesis, mitochondrial oxygen consumption is needed for the transition of differentiating cells from glycolytic to oxidative metabolism and the clonal expansion of preadipocytes, and then more energy is needed to acquire the typical metabolic phenotype of mature adipocyte [3].

Mature adipocytes make up only 20–30% of the total number of cells in adipose tissue. The remaining cells are made up of stromal vascular fraction and belong to the immune, epithelial, vascular, and stromal cells [4]. Besides storage and distribution of energy, adipose tissue contributes to the regulation of systemic energy metabolism by means of adipokines secretion such as adiponectin, leptin, resistin, interleukin-6 (IL-6), and tumor necrosis factor α. The secretion of adipokines enables autocrine, paracrine, endocrine, and cross talk communication with other organs [5]. Physiological production of adipokines requires intact cellular machinery of mature adipocyte, in particular mitochondrial respiration and balance between lipogenesis and lipolysis. Dysfunctional secretion of adipokines and free fatty acids induces an inflammatory response that is supposed to be the basis of peripheral insulin resistance [6]. These processes are linked to the specific distribution and accumulation of visceral fat, its morphological and inflammatory restructure, which are the main causes of metabolic complications, like diabetes mellitus type 2 and increased cardiovascular risk, even in a population with a normal body weight [7,8].

According to the last definition of the European Commission, obesity is depicted as a chronic relapsing disease, which in turn acts as a gateway to a range of other non-communicable diseases, such as diabetes, cardiovascular diseases, and cancer. Over the last forty years, the prevalence of obesity is increasing worldwide, achieving pandemic levels [9]. Many epidemiological and experimental studies suggest that obesity and associated dysfunction of adipose tissue might be a consequence of several interconnected causes such as genetic, environmental, and social factors [10,11].

The obesogenic environment is crucial in this trend. One of the possible characteristics of the obesogenic environment is chronic exposure to environmental contaminants, especially organochlorines, like pesticide 1,1,1-trichloro-2,2,-bis[p-chlorophenyl]ethane (DDT) [12]. It has been documented that DDT and its metabolite 2,2-bis (4-chlorophenyl)-1,1-dichlorethylenedichlorodiphenyldichloroethylene (p,p′-DDE) are associated with the increased risk of obesity and type 2 diabetes mellitus, and therefore they are called "metabolic disruptors" or "obesogens" [13–16]. Although production of DDT was banned in the late 1970s, its metabolites, especially p,p′-DDE can still be detected in human serum and samples rich in fat, such as breast milk [17,18].

DDE is accumulated and stored in lipophilic tissues, especially in adipose tissue. The variability of the stored amount of DDE in adipose tissue can range over several orders of magnitude, depends on dietary exposure and on individual disposition to store these substances [19,20]. For example, in the Smeds and Saukko study, DDE concentration levels in human adipose tissue ranged from 3.5 to 3229 ng/g lipids [13]. That serum concentration levels in men may exceed 3000 ng/g lipids was also confirmed [14]. These high values of serum concentration, based on lipid weight conversion correspond to 0.1 μM DDE. Concentration in the adipose tissue could be even one order higher.

The obesogenic action of these compounds disrupts homeostatic control over energy balance leading to overabundance in the metabolic pathways involved in energy storage over those that are responsible for metabolic energy expenditure flow [21]. Increasing evidence suggests that mitochondria might be a key player in the development of obesity. Multiple experimental studies demonstrated the inhibitory effects of organochlorines on mitochondrial oxygen consumption in the liver, brain, or skeletal muscle [22,23]. The impact

of long-lasting DDE exposure on mitochondrial respiration of differentiating adipocytes has not been studied yet [24].

In our study, we employed human adipose-derived mesenchymal stem cells (hADMSCs) committed to adipogenesis. The effects of p,p′-DDE in concentrations 1 µM and 10 µM on differentiating adipocyte mitochondrial oxygen consumption, citrate synthase activity, and mitochondrial membrane potential were evaluated on days 4, 10, and 21 of adipogenesis. In addition, lipid accumulation and mitochondrial quality were assessed to verify the phenotype of differentiating cells.

2. Materials and Methods

2.1. Cell Culture and Differentiation In Vitro

Human adipose-derived mesenchymal stem cells were isolated from a female donors' subcutaneous adipose tissue, purchased from Thermo Fisher (Thermo Fisher Scientific, Carlsbad, CA, USA) and cultivated. The cells were seeded at 1×10^5 cells/cm^2 and cultured in Petri dishes (TPP Techno Plastic Products, Trasadingen, Switzerland) in commercially available culture medium MesenPRO RS™. The medium was supplemented with MesenPRO RS™ Growth Supplement with reduced serum level (2%), 1% L-glutamine, and 1% gentamicin (all Thermo Fisher Scientific, Carlsbad, CA, USA).

After reaching 80% confluence, the culture medium was changed to four different differentiation media: (A) pure differentiation medium (DM); (B) differentiation medium with dimethyl sulfoxide (DMSO); (C) differentiation medium with DMSO and p,p′-DDE, in final concentration 1 µM (DDE 1 µM); (D) differentiation medium with DMSO and p,p′-DDE in final concentration 10 µM (DDE 10 µM). The pure differentiation medium contained StemPro® Adipogenesis Differentiation Basal Medium with StemPro® Adipogenesis Supplement and 1% gentamicin (all Thermo Fisher Scientific, Carlsbad, CA, USA). DDE concentrations 1 µM and 10 µM were chosen because of their most common use in in vitro studies. DMSO, which is commonly used as a solvent of lipophilic compounds for in vitro cell experiments, is generally considered nontoxic up to 0.05% concentration (*v*/*v*) [18]; however, some studies suggest that this solvent can even promote proliferation at least in some cell lines [25]. On that account, we first compared the differentiation process under DM alone with the differentiation medium with DMSO in concentration used in our experiments, i.e., 0.019%. We have found that the addition of DMSO affected the process of differentiation, since the cell count per well measured using NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) as described below displayed a slightly different pattern in the course of adipogenesis. Thus, all our results acquired from DDE-exposed cell cultures were compared separately to the cells differentiating in the same medium with or without DMSO. A summary scheme of the experiment is shown in Figure 1.

The medium was changed every 3 days up to a total incubation time of 21 days. The cells were maintained and cultured into differentiated adipocytes under 5% CO_2 atmosphere at 37 °C [26]. The independent experimental number for each method was 12 except in high-resolution respirometry. With the aim to prove that the experimental system was running as expected, we monitored the process of adipogenesis by Oil Red O staining and fluorescent probe staining methods.

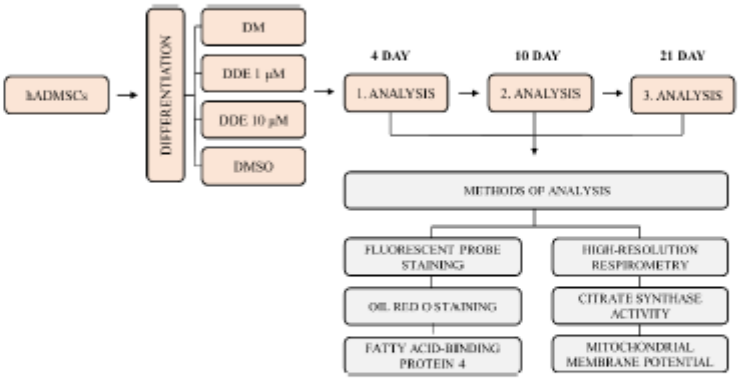

Figure 1. Summary scheme of the experiment.DM: pure differentiation medium; DDE 1 μM: differentiation medium with added p,p′-DDE, which was dissolved in dimethyl sulfoxide (DMSO); DDE 10 μM: differentiation medium with added p,p′-DDE, which was dissolved in DMSO; DMSO: differentiation medium (DM) with DMSO only.

2.2. High-Resolution Respirometry

Mitochondrial respiration of intact adipocytes on days 4 ($n = 6$ per group; i.e., DM, DMSO, DDE 1 μM, DDE 10 μM), 10 ($n = 6$) and 21 ($n = 6$) of differentiation was analyzed in the StemPro® Adipogenesis Differentiation medium using high-resolution respirometry in a 2-chamber oxygraph O2k (Oroboros Instruments, Innsbruck, Austria). The negative time derivative of the oxygen concentration in the chamber was calculated online to determine oxygen consumption (DatLab software, version 7.3.0.3, Oroboros, Austria). The cells were applied into the open precalibrated oxygraph chambers, stirred at 350 rpm and a sample of 15 μL of the cell suspension was aspirated to count the cells in the Bürker hemocytometer. After closing the oxygraph chambers, a substrate-uncoupler-inhibitor titration (SUIT) protocol for intact cells was used to determine the standard respiratory states: ROUTINE (ROUT; R)—oxygen consumption at the physiological coupled state; LEAK (L; injection of 2.5 μmol/L oligomycin)—non-phosphorylating resting state of respiration to compensate for the proton leak when ATP synthase is not active; the electron-transfer-system capacity (ETSC; E) was assessed after titrations of trifluorocarbonylcyanide phenylhydrazone (FCCP; 0.05 μmol/L titration steps) to reach maximal oxygen consumption in the noncoupled state; and ROX, residual oxygen consumption indicating oxidative side reactions remaining after inhibition of the electron transfer pathway (0.5 μmol/L rotenone and 2.5 μmol/L antimycin A) [21]. Oxygen consumption was expressed in pmoL $O_2/(s \cdot 10^6$ cells) and corrected to ROX and instrumental background. The representative scheme of the experiment on intact cells is shown in Figure 2.

In addition, the following control parameters were calculated: L/E coupling control ratio as an index of uncoupling; R/E control ratio showing how close ROUT operates to ETSC; E-R reserve capacity reflecting the difference between noncoupled and coupled respiration; R-L or net routine capacity related to the cellular ATP production.

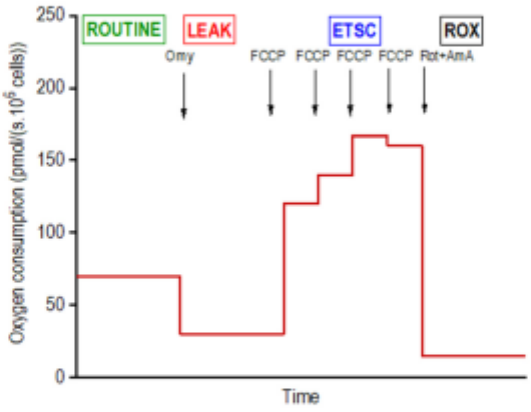

Figure 2. Design of a titration protocol for measuring the mitochondrial oxygen consumption in intact differentiating adipocytes. For the concentrations of inhibitors and uncoupler utilized and definition of respiratory states, see Methods.

2.3. Citrate Synthase Activity

The determination of citrate synthase activity was used to estimate mitochondrial content in the samples from each oxygraph chamber. The assay medium for citrate synthase activity consisted of 0.1 mmol/L 5,5-dithio-bis- (2-nitrobenzoic) acid, 0.25% triton-X, 0.5 mmol/L oxaloacetate, 0.31 mmol/L acetyl coenzyme A, 5 µmol/L EDTA, 5 mmol/L triethanolamine hydrochloride, and 0.1 mol/L tris-HCl, pH 8.1. One hundred microliters of the mixed and homogenized chamber content were added to 900 µL of the medium. The rate of absorbance change was measured spectrophotometrically at 412 nm and 30 °C over 200 s.

2.4. Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Mitosciences, Abcam, Cambridge, UK). The cells were seeded at 1×10^4 cells on a dark 96-well plate and cultured as explained earlier. The MMP was evaluated on days 4 ($n = 12$), 10 ($n = 12$), and 21 ($n = 12$) of differentiation. The cells were washed once with phosphate buffered saline (PBS) and incubated with JC-1 dye (1 µM) at 37 °C for 10 min. Then the cells were rinsed twice with PBS and were analyzed on a fluorescence spectrophotometer (Synergy HT, BioTek, Winooski, VT, USA) at excitation 475 nm and emission 530/590 nm. The red/green fluorescence intensity ratio was determined to evaluate MMP.

2.5. Oil Red O, Fluorescent Probe Staining, and Fatty Acid-Binding Protein 4

All these methods have been used to prove that the experimental system was performing as expected.

2.5.1. Oil Red O Staining

Oil Red O is a fat-soluble dye that stains neutral triglycerides and lipids [27]. Intracellular triglyceride droplets of hADMSCs were stained with Oil Red O solution on day 4, 10, and 21. The sample preparation procedure was as follows: medium was removed, each well was rinsed twice in PBS, and fixed in 4% formaldehyde for 1 h at room temperature. The cells were then rinsed twice with distilled water and stained with Oil Red O solution (0.5 g of Oil Red O powder dissolved in 100 mL of isopropanol). This solution was blended with distilled water in the ratio 3:2. Cells were incubated with this solution for 15 min

at room temperature, then washed twice with distilled water and visualized using the Olympus CX41 microscope (Olympus, Tokyo, Japan) connected to a digital camera.

Figure 3 shows the intracellular fat droplets during differentiation in all four types of the media.

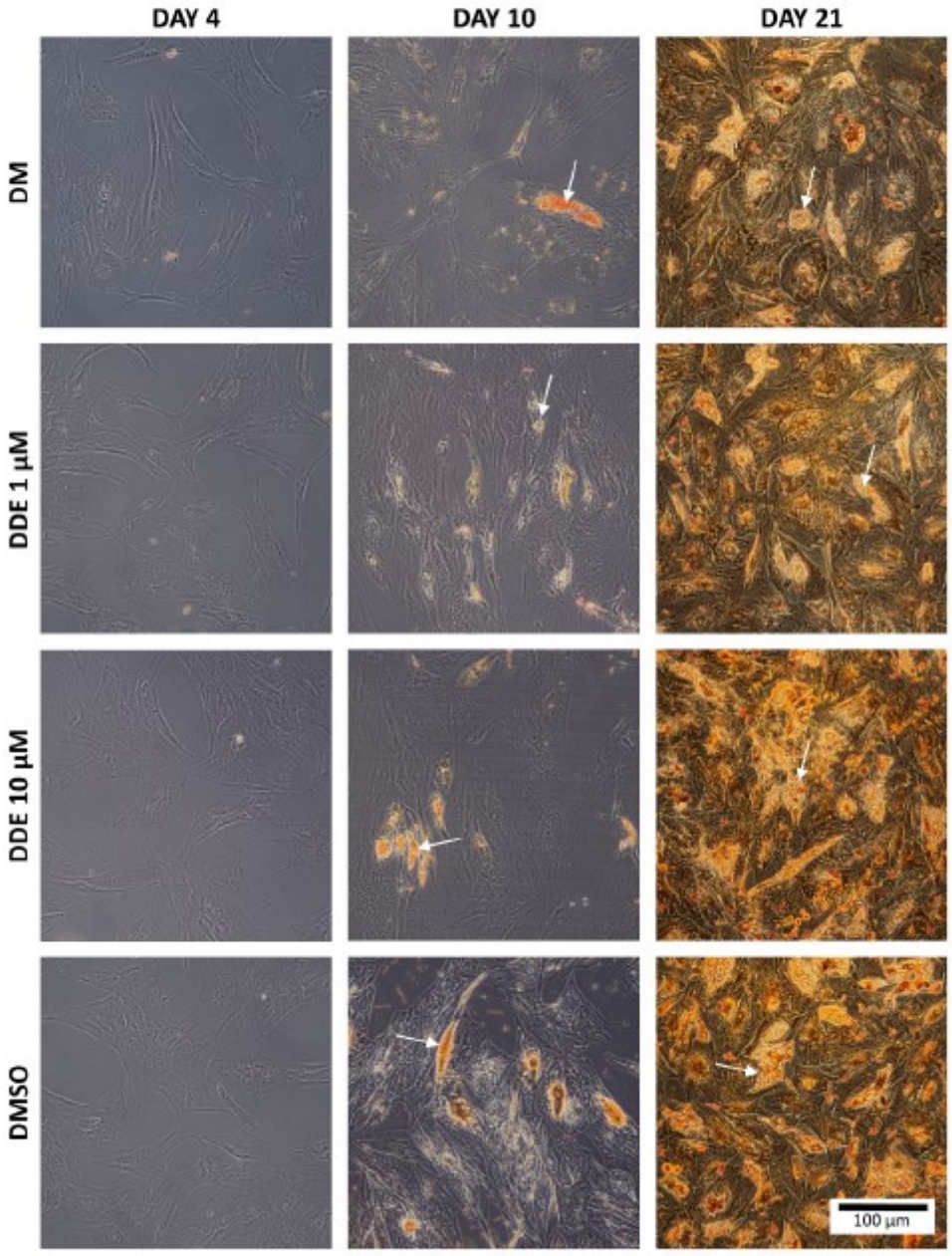

Figure 3. The comparison of fat in each environment. The arrows show examples of fat droplets. (Oil Red O staining).

2.5.2. Fluorescent Probe Staining

Fluorescent probes, MitoTracker™ Red CMXRos and NucBlue™ Live ReadyProbes Reagent (both Molecular Probes, Eugene, OR, USA) were used to visualize the mitochondria and nuclei of cells. MitoTracker™ passively passes into mitochondria and accumulates there. NucBlue™ Reagent contains Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole) which emits a blue fluorescence when bound to DNA (Figure 4).

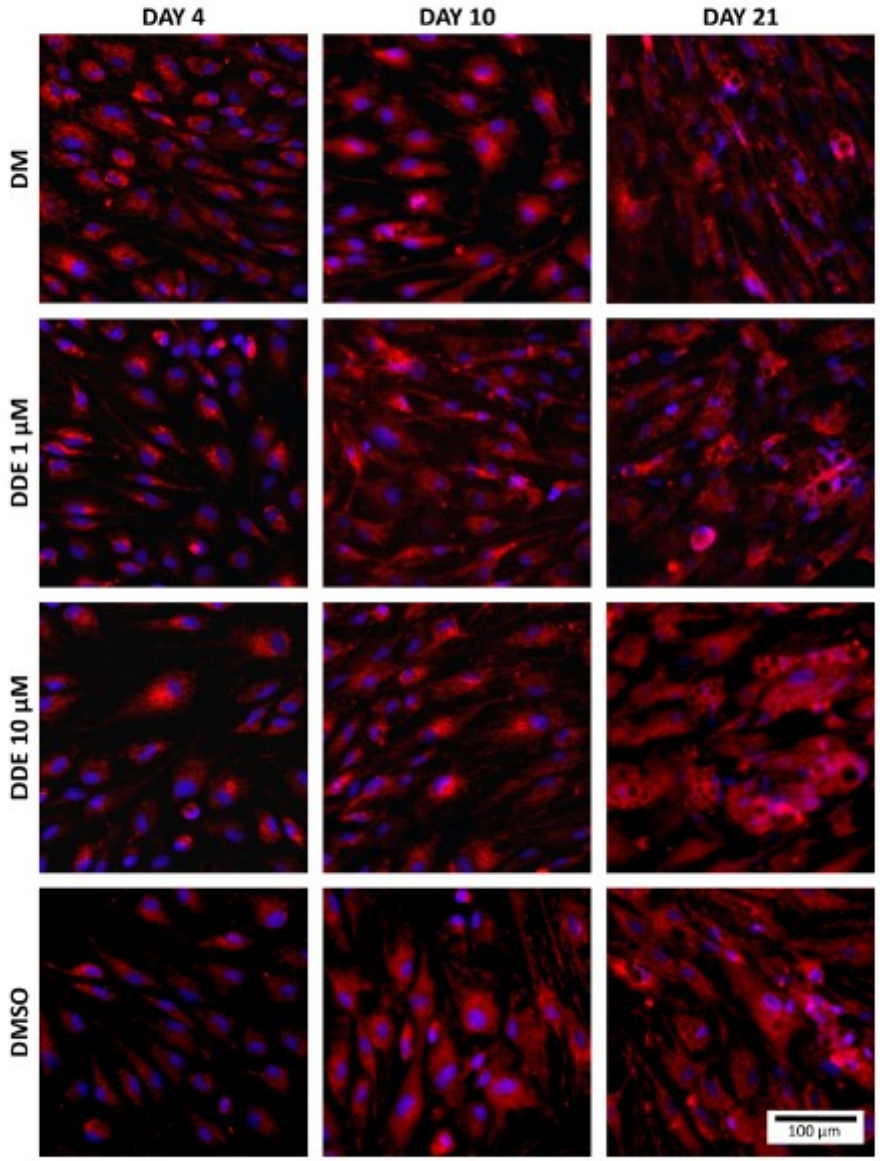

Figure 4. Staining of mitochondria (red) and nuclei (blue) in each environment (fluorescent probes, MitoTracker™ Red CMXRos and NucBlue™ Live ReadyProbes Reagent were used to visualize the mitochondria and nucleus of cells).

The cells were first stripped of differentiation medium, which was replaced with special medium for live cell imaging—Live Cell Imaging Solution (Molecular Probes, Eugene, OR, USA). Subsequently, Mitotracker™ was added to the medium (final concentration 100 nM) and two drops per millilitre of NucBlue™ were added too. Cells were incubated in the dark for 30 min and then visualized by the Hamamatsu Orca-ER camera mounted on the Olympus IX 81 inverted microscope at 200× magnification (Olympus, Tokyo, Japan).

2.5.3. Fatty Acid-Binding Protein 4

Fatty acid-binding protein (FABP4) is highly expressed in adipocytes and consists of about 1% of all soluble proteins in adipose tissue [28]. On day 21, the adipogenic marker FABP4 was measured to confirm the presence of adipocytes.

Cells were washed in phosphate buffered saline (PBS), fixed for 60 min in 4% formaldehyde with PBS at room temperature, and permeabilized in PBS containing 0.3% Triton X-100 for 15 min followed by blocking in PBS with 1% bovine serum albumin (BSA) and 10% normal donkey serum at room temperature for 60 min. After blocking, cells were incubated with anti-mFABP4 antibody (anti-mouse Fatty Acids Binding Protein 4, R&D Systems, Inc., Minneapolis, MN, USA) working solution (PBS containing 0.03% Triton X-100, 1% BSA, 10% normal donkey serum and anti-mFABP4 in final concentration 10 µg/mL) overnight at 2–8 °C. After three 5-min rinses in PBS with 1% BSA, cells were incubated for 1 h in NL557-conjugated anti-goat secondary antibody (R&D Systems, Inc., Minneapolis, MN, USA) diluted 1:200 in 1% BSA in PBS in the dark for 60 min at room temperature. The coverslips were washed, placed on microscope slides with a mounting medium (ProLong Gold Antifade Mountant with DAPI, Molecular Probes, Eugene, OR, USA) and visualized using the Hamamatsu Orca-ER camera mounted on the Olympus IX 81 inverted microscope at 200× magnification (Olympus, Tokyo, Japan).

2.6. Data Analysis and Statistics

The data were processed with the use of the statistical software MATLAB Statistics Toolbox (MathWorks Inc., Natick, MA, USA) and OriginPro 2017 (OriginLab Corp., Northampton, MA, USA). After testing for normality of distribution (Shapiro Wilk test), normally distributed data were analyzed using two-way ANOVA followed by the Tukey post hoc test. Non-normally distributed data were processed by log-transformation and then analyzed accordingly. In addition, the differences between groups that required transformation or data that could not reach normal distribution were analyzed using the Wilcoxon rank sum and Friedman statistical tests. p-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. High-Resolution Respirometry

As expected, in the control samples (DM and DMSO), the routine respiration increased between days 4 and 10 of the experiment by ~30%. However, the differences did not reach statistical significance (Figure 5A). In contrast, routine oxygen consumption observed in DDE 1 µM samples on day 10 of adipogenesis was ~50% higher than on day 4 ($p < 0.0001$) and became significantly different also when compared to DDE 10 µM cells. DDE 10 µM adipocytes did not display any increase in the state ROUT between days 4 and 10 of adipogenesis. In cells differentiating in the control media only (DM and DMSO), routine respiration remained nearly the same on day 21. DDE 10 µM cells had higher routine oxygen consumption on day 21 compared to day 4. In adipocytes differentiating in the presence of 1 µM DDE, routine oxygen consumption further increased on day 21 of the experiment and became significantly different compared to both DMSO and DM controls (Figure 5A). The LEAK state, i.e., oxygen consumption after ATP synthesis inhibition by oligomycin gradually increased from day 4 to 21 in the control (DMSO) and DDE 1 µM samples reaching significant difference between days 4 and 21 of the experiment. In DDE 10 µM adipocytes, the leak respiration was very similar on days 4 and 10 (11.6 ± 2.9 and

10.1 ± 3.2 pmol/s · 10^6 cells, respectively) and then increased reaching significant difference between days 10 and 21. No significant difference between the treatments was noted on days 4, 10, or 21 of the experiment (Figure 5B). ETSC did not differ between days 4 and 10 and then tended to decline resulting in a significant difference between the DMSO control and DDE 1 µM adipocytes on day 21 of the experiment. In addition, the ETSC state of DMSO control cells was significantly lower on day 21 compared to day 10 (Figure 5C).

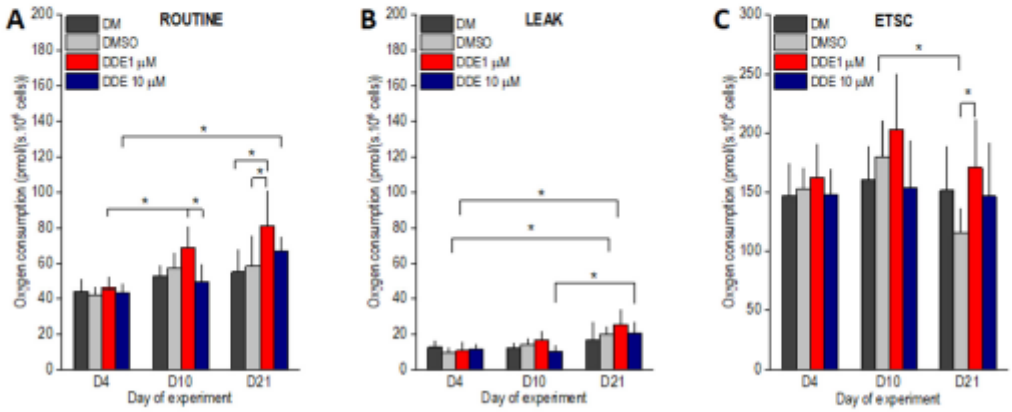

Figure 5. (**A**–**C**) ROUTINE, LEAK and uncoupled (ETSC) oxygen consumptions of adipocytes differentiating in media containing no additive (DM) or DMSO, DMSO and 1 µM p,p′-DDE, and DMSO and 10 µM p,p′-DDE on days 4, 10, and 21 of the experiment. Significant differences * $p < 0.05$ (Two-way ANOVA followed by a post-hoc Tukey test).

ATP-linked oxygen consumption, i.e., the R-L state, slightly increased between days 4 and 10 in all groups of differentiating adipocytes and then remained stable. Significant differences between days 4 and 10 of adipogenesis were reached only in DDE 1 µM and DMSO-control samples. On day 21 of the experiment, R-L was significantly higher in DDE 1 µM compared to DM and DMSO control groups. Reserve respiratory capacity, i.e., the E-R state, decreased between days 10 and 21; however, this decline was significant only in DMSO adipocytes. The L/E coupling control ratio significantly increased in all DMSO-containing samples on day 21, suggesting that the extent of uncoupling was similar in controls and DDE-treated adipocytes. Similarly, the R/E control ratio increased to a comparable extent in all groups of adipocytes on day 21 of the experiment, documenting that in the later stages of differentiation, routine respiration operates closer to ETSC (Figure 6).

Citrate synthase activity ranged between 10.61 ± 5.26 to 18.32 ± 4.74 mIU/10^6 cells in DMSO control cells on day 21 and DDE1 adipocytes on day 10, respectively. No significant difference was noted between individual groups on any day of the experiment nor between adipocytes after the same intervention in the course of the experiment. Data not shown.

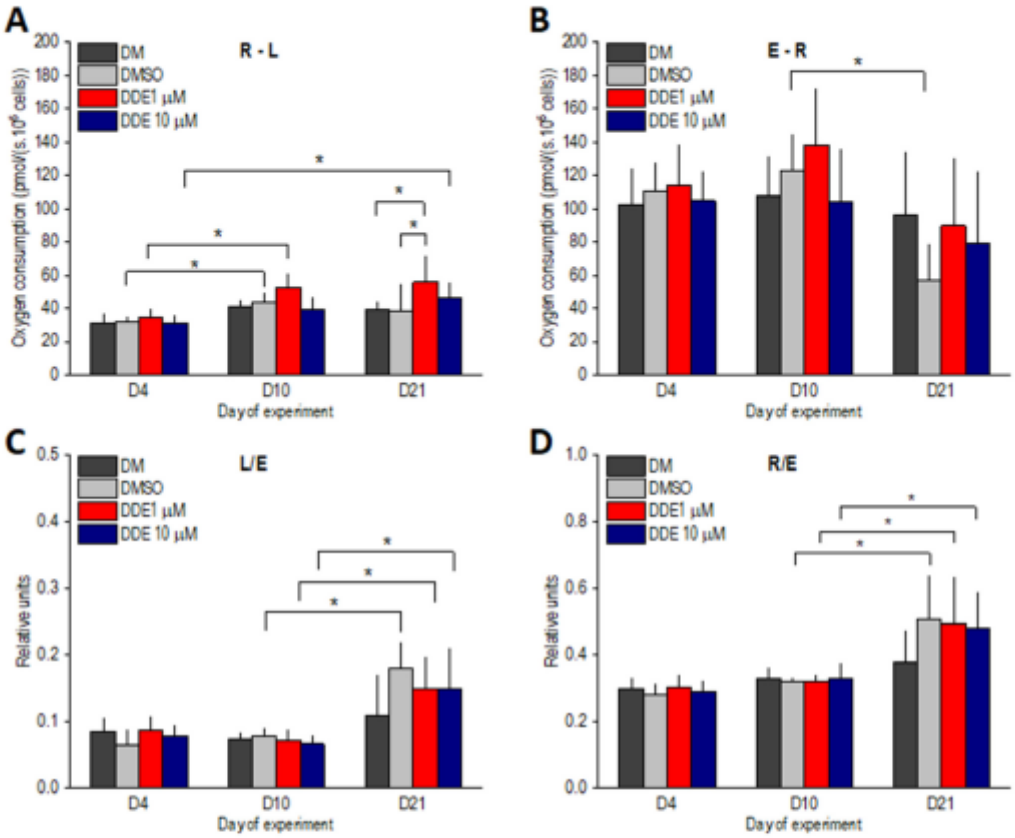

Figure 6. (**A**,**B**) ATP-related oxygen consumption (R-L) and reserve ETS capacity (E-R) in adipocytes differentiating in media containing no additive (DM) or DMSO, DMSO and 1 µM p,p'-DDE, and DMSO and 10 µM p,p'-DDE on days 4, 10, and 21 of the experiment. (**C**,**D**) Flux control ratios L/E and R/E. Significant differences * $p < 0.05$ (Friedman and Wilcoxon rank sum tests).

3.2. Mitochondrial Membrane Potential

MMP is one of the key parameters of mitochondrial function and serves as an indicator of cell health—regulation of ATP synthesis, ROS production, calcium sequestration, etc. In healthy cells, JC-1 dye enters the mitochondria and forms red aggregates. Mitochondria in cells with low mitochondrial potential do not form aggregates and remain in monomeric form with green fluorescence. We compared JC1 staining in unaffected adipocyte differentiation and in differentiation under the influence of DMSO and DMSO with DDE. In all cases, we observed a gradual decrease in the red/green ratio during differentiation, which corresponds to previous findings [3,29]. On the fourth day of differentiation, a significant decrease in MMP was observed in DDE-affected cells at both 1 µM and 10 µM concentrations compared to medium containing DMSO alone. In cells affected with 1 µM DDE, there was a significant increase in red/green ratio on day 21 of differentiation compared to cells exposed to DMSO alone (Figure 7).

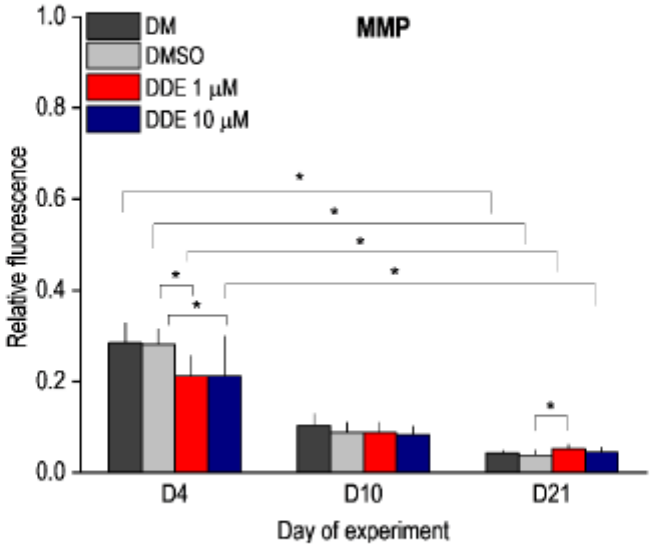

Figure 7. Mitochondrial membrane potential (MMP) during adipocyte differentiation in media containing no additive (DM) or DMSO, DMSO, and 1 μM p,p'-DDE, and DMSO and 10 μM p,p'-DDE on days 4, 10, and 21 of the experiment. Significant differences * $p < 0.05$ (Friedman and Wilcoxon rank sum tests). On the fourth day of differentiation, a significant decrease in MMP was observed in DDE-affected cells at both 1 μM and 10 μM concentrations compared to medium containing DMSO alone. In cells affected with 1 μM DDE, there was a significant increase in MMP on day 21 of differentiation compared to control cells exposed to DMSO alone.

4. Discussion

In our study, we monitored chronic DDE exposure at two different concentrations to a developing adipocytes differentiating from hADMSCs. We found that the long-lasting treatment with DDE in a concentration of 1 μM in contrast to 10 μM resulted in statistically significant deviations in mitochondrial respiration compared to control cells. The characteristic pattern of changes in mitochondrial respiration during adipogenesis was not impaired by lower DDE concentration showing a typical gradual increase in the routine and leak respirations of maturing adipocytes [3]. However, on day 21 of the experiment—i.e., in adipocytes regarded as fully differentiated—higher mitochondrial membrane potential, higher resting mitochondrial oxygen consumption (routine respiration), and higher ATP-related respiration (R-L) were observed in DDE 1 μM samples compared to relevant controls. Our results suggest that lower concentrations of the pollutant during chronic exposure may more significantly modulate physiological processes, in this case probably through an endocrine disrupting effect. Such a finding might seem surprising since the majority of studies dealing with the impact of organochlorines on mitochondrial functions reported impaired oxygen consumption of the tissues studied [27–31]; reviewed in [12]. However, it should be noted that the concentrations of DDT and DDE used in these studies were relatively high and their effects were analyzed mainly on the liver and muscle mitochondria after a single dose exposure. The design of our experiment differs in the chronicity of exposure, lower concentrations of the pollutant used, the continuous action of DDE on the whole process of adipocyte differentiation until maturation, and the type of affected cells.

Appropriate mitochondrial function in general and oxidative phosphorylation in particular is essential for ATP production and the whole-body energy homeostasis. Mitochondrial dysfunction has been implicated in the development of many pathological

conditions associated with obesity as a result of imbalance between food intake and energy expenditure, such as type 2 diabetes mellitus. Nevertheless, mitochondrial dysfunction does not necessarily mean that mitochondrial oxygen consumption and ATP production should be decreased in all tissues involved in the regulation of energy homeostasis. Tissue-specific control of mitochondrial respiration was demonstrated in obese diabetic mice displaying impaired mitochondrial respiration in the liver and oxidative skeletal muscle, but enhanced oxygen consumption in glycolytic skeletal muscle [32]. Another study reported increased mitochondrial oxygen consumption in the brown fat of obese mice [33]. In addition, adipocyte-specific decrease in oxidative phosphorylation affected systemic energy homeostasis and protected against the development of obesity and insulin resistance in mice fed a high-fat diet [34]. In the study conducted by Böhm et al. (2020), enhanced mitochondrial respiration of adipocytes isolated from obese insulin-resistant donors was attributed to adaptation of the cellular metabolism to the increased amount of fuels associated with insulin resistance [35].

In our study, the supply of metabolic substrates was the same in all experimental groups; however, increased ATP demand in DDE-treated adipocytes could be related to the promoting effect of DDE and similar pollutants on the de novo synthesis of lipids and their accumulation [36]. The negative effects of DDT/DDE on thermogenic proteins expression and substrate transport/utilization in adipocytes were also documented, which could lead to an increased ATP need to compensate for compromised fuel transport or heat dissipation [37]. A recent study suggests that impaired thermogenesis in DDT-affected adipocytes could be caused by targeting mechanisms upstream of adipose tissue without the necessity to compromise the expression of uncoupling proteins [38]. In this study, the leak respiration needed to compensate for proton leak, electron slip, and cation cycling increased in the course of adipocyte differentiation in all groups to a similar extent, without reaching any significant difference between DDE-treated and control groups on day 21.

The excess E-R capacity (respiratory reserve) was the only parameter compromised by the long-lasting exposure to DMSO (between days 10 and 21 of differentiation). Nevertheless, the same trend was observed in DDE-treated adipocytes on day 21 verifying our previous finding that in the later stages of differentiation, the routine respiration could be increased only on the account of the total electron-transporting capacity of mitochondria [3]. In addition, the ratio of routine to maximal respiration (R/E) was nearly the same in DMSO-control and DDE-treated adipocytes on day 21 of the experiment and in all these groups it reached significantly higher values than on day 10 of differentiation.

Another interesting finding of this study is the fact that at higher concentration, i.e., 10 µM, DDE seemed to have no effect, since on day 21, no significant differences were revealed between DDE 10 µM and control adipocytes. However, DDE 10 µM–exposed differentiating adipocytes displayed a later onset of changes in mitochondrial parameters associated with normal adipogenesis, i.e., an increase in routine respiration and ATP-linked oxygen consumption. Such "bidirectional" action of various pollutants on the cellular functions is not a new finding and makes the research into the putative effects of endocrine disruptors more complicated [39].

Thus, evaluating the impact of DDE on mitochondrial functions in human differentiating adipocytes, adipogenesis can be attacked in two ways: (i) at higher DDE concentrations by slowing down the differentiation process, (ii) at lower chronic DDE levels by increased mitochondrial respiration and ATP generation, which then could lead to disturbances in energy homeostasis.

Mitochondrial dysfunction of the adipocyte may then be involved in the pathogenesis of obesity-related metabolic diseases, such as diabetes mellitus, in particular if the supply of metabolic substrates is increased simultaneously. Overexposure of cells to saturated fatty acids, which is also associated with higher exposure to DDT and DDE from food of animal origin might aggravate mitochondrial dysfunction of other tissues and contribute to disturbances of the whole-body energy balance [40].

5. Study Limitations

This study addressed the impact of persistent organochlorine pollutant p,p′-DDE on mitochondrial respiration in a single cell model. However, the adipocytes differentiating from hADMSCs have only limited survival time and cannot provide complex insight into impaired regulation of the functions of adipose tissue exposed to potential obesogens for the whole life. In future studies, long-lasting exposure to the pollutant should be extended to in vivo models to verify the putative disrupting effect of the compound on energy homeostasis and to specify the mechanism by which it could contribute to obesity and related diseases.

6. Conclusions

Elucidating the cellular and molecular mechanisms of DDE obesogenic effects is critical to understand the putative causal relationship of DDE to obesity and its metabolic complications.

This study focused on the impact of DDE on the metabolic characteristic of hADMSCs in the whole course of differentiation (21 days). Its results show that subtle sequelae of DDE chronic action could be observed at the end of differentiation, i.e., after long-lasting exposure. Maturing adipocytes "adapted" to continuous supply of the pollutant in lower doses (1 μM), displayed higher basal mitochondrial respiration and ATP-linked oxygen consumption along with impaired uncoupling reflected in higher mitochondrial membrane potential that might interfere with efficient heat dissipation. A higher concentration of the pollutant slows down the differentiation process, as documented by the later onset of the increase in ATP-linked oxygen consumption.

In conclusion, the hADMSCs in vitro model of differentiation is suitable to study the impact of chronic DDE exposure on different features of adipogenesis. The long-lasting action of DDE seems to result in metabolic reprogramming of adipocytes that might contribute to the obesogenic effect of the pollutant studied.

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Data Availability Statement: Data available on request due to privacy restrictions. The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the co-ownership of multiple workplaces.

Conflicts of Interest: The authors declare no conflict of interest.

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Mitochondrial Respiration of Adipocytes Differentiating From Human Mesenchymal Stem Cells Derived From Adipose Tissue

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Summary

Burden of obesity is increasing in the contemporary world. Although multifactorial in origin, appropriate mitochondrial function of adipocytes emerges as a factor essential for healthy adipocyte differentiation and adipose tissue function. Our study aimed to evaluate mitochondrial functions of human adipose-derived mesenchymal stem cells committed to adipogenesis. On days 0, 4, 10, and 21 of adipogenesis, we have characterized adipocyte proliferation and viability, quantified lipid accumulation in maturing cells, performed qualitative and quantitative analysis of mitochondria, determined mitochondrial respiration of cells using high-resolution respirometry, and evaluated mitochondrial membrane potential. In the course of adipogenesis, mitochondrial oxygen consumption progressively increased in states ROUTINE and E (capacity of the electron transfer system). State LEAK remained constant during first days of adipogenesis and then increased probably reflecting uncoupling ability of maturing adipocytes. Citrate synthase activity and volume of mitochondrial networks increased during differentiation, particularly between days 10 and 21. In addition, lipid accumulation remained low until day 10 and then significantly increased. In conclusion, during first days of adipogenesis, increased mitochondrial respiration is needed for transition of differentiating cells from glycolytic to oxidative metabolism and clonal expansion of preadipocytes and then more energy is needed to acquire typical metabolic phenotype of mature adipocyte.

Key words

Mesenchymal stem cells • Adipocyte • Adipogenesis • High-resolution respirometry • Mitochondria

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Introduction

Obesity represents an increasing problem of the contemporary world. It leads to various chronic morbidities like Type 2 diabetes mellitus, hypertension, atherosclerosis, heart disease, stroke, cancer, infertility etc. (Cai *et al.* 2010, Castro *et al.* 2014, Luna-Luna *et al.* 2015, Mission *et al.* 2015, Zimmet *et al.* 2001). Caloric intake exceeding energy expenditure produces a metabolic state that promotes hypertrophy and hyperplasia of adipocytes (Shepherd *et al.* 1993). It is supposed that the increase in adipocyte number is triggered by signaling factors that induce the commitment of pluripotent mesenchymal stem cells residing in the vascular stroma to the adipocyte lineage. When committed, preadipocytes are subjected to mitotic clonal expansion undergoing two or three cell divisions and then they gradually acquire typical adipocyte metabolic and

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morphological characteristic in the process of differentiation (Tang and Lane 2012). All three stages are essential for the development of obesity and they are tightly linked to the increased energy intake over long time periods (Qiao *et al.* 2019, Shepherd *et al.* 1993, Spiegelman and Flier 1996).

Appropriate function of white adipose tissue is dependent on energy provided by mitochondria (Kusminski and Scherer 2012). It has been also suggested that adipocyte differentiation is at least partly driven by mitochondrial oxidative phosphorylation (Luz *et al.* 2019, Zhang *et al.* 2013).

To study adipogenesis *in vitro*, 3T3-L1 preadipocyte cell line originally derived as a subclone of the 3T3 mouse embryonic fibroblast cell line is widely used in experimental practice and some data are also available on mitochondrial respiration of these cells (Morrison and McGee 2015). However, the translation potential of the experimental results acquired from these cells has been challenged by several important issues (Sadie-Van Gijsen 2019).

Human cell models include various types of stem cells that are able to differentiate into adipocytes, namely adipose tissue derived mesenchymal stem cells that allow investigation of all phases of adipocyte formation. Despite concerns expressed by some investigators (Berry *et al.* 2014), these cells represent a more relevant model suitable for research in adipocyte biology than most rodent cell lines (Sadie-Van Gijsen 2019).

The aim of our study was to extend earlier observations of other researchers in the field of mitochondrial physiology of differentiating human adipocytes. We have used human adipose-derived mesenchymal stem cells committed to adipogenesis and on days 0, 4, 10 and 21 of differentiation, we have characterized their proliferation and viability, quantified lipid accumulation in maturing cells, performed qualitative and quantitative analysis of mitochondria, determined mitochondrial respiration of cells using high-resolution respirometry, and evaluated mitochondrial membrane potential.

Methods

Cell culture and differentiation

Human adipose-derived mesenchymal stem cells (hADMSCs), isolated from a female donor from subcutaneous adipose tissue and characterized by flow cytometry were purchased from Thermo Fisher (Thermo Fisher Scientific, Carlsbad, CA, USA) and cultivated as described previously (Mullerova *et al.* 2017). Briefly, the cells were seeded at 1×10^5 cells and cultured in Petri dishes (TPP Techno Plastic Products, Trasadingen, Switzerland) in commercially available culture medium MesenPRO RS™ Medium supplemented with MesenPRO RS™ Growth Supplement with reduced serum level (2 %), 1 % L-glutamine and 1 % gentamicin (all Thermo Fisher Scientific, Carlsbad, CA, USA).

After 80 % confluence was reached, the hADMSCs were cultured for adipogenic differentiation in culture plates according to the manufacturer's instructions in StemPro® Adipogenesis Differentiation Basal Medium (DM) with StemPro® Adipogenesis Supplement and 1 % gentamicin (all Thermo Fisher Scientific, Carlsbad, CA, USA). The medium was changed every 3 days up to a total incubation time of 21 days. The cells were maintained and cultured into differentiated adipocytes under 5 % CO_2 atmosphere at 37 °C.

Cell proliferation

Human adipose-derived stem cells were seeded at 5000 per well on the bottom of 96 dark well plate and treated as described above. Samples were labeled on days 0, 4, 10, 21 using NucBlue® Live ReadyProbes® Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). All samples were scanned using the Olympus IX83 (Olympus, Tokyo, Japan) equipped with a VisiScope Live Cell Imagine system at 100× magnification. The images were analyzed using the ImageJ (FIJI) software.

Cell viability

PrestoBlue™ cell viability reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) was used to evaluate the viability and proliferation of cells (Dejmek *et al.* 2019). The reagent uses mitochondrial activity to reduce the non-fluorescent blue resazurin to the fluorescent pink resofurin. PrestoBlue™ reagent (10 µl) was added to 90 µl medium with cells on 96 dark well plate. Cells were incubated for 10 min at 37 °C. The bottom-read fluorescence was read at 560 nm (excitation) and 590 nm (emission) by Synergy™ HT microplate reader (BioTek, Winooski, VT, USA).

Differentiation and maturation of adipocytes

1. Oil Red O staining

Human adipose-derived stem cells were stained using Oil Red O solution which stains intracellular

triglyceride droplets. Medium was removed from each well, cells were twice rinsed in phosphate buffered saline (PBS) and fixed in 4 % formaldehyde prepared in PBS for 1 h at room temperature. Then the cells were washed twice in distilled water and stained in Oil Red O solution. This solution was prepared dissolving 0.5 g of Oil Red O powder (Sigma-Aldrich, Prague, Czech Republic) in 100 ml of isopropanol (Sigma-Aldrich, Prague, Czech Republic), filtered with absorbent paper and blended in the ratio 3:2 – three parts of 0.5 % Oil Red O solution and two parts of distilled water. The cells were incubated for 15 min with the Oil Red O solution at room temperature, twice washed in distilled water to remove unbound dye and the cells were then visualized using an inverted microscope Olympus CX41 (Olympus, Tokyo, Japan) connected to a digital camera.

2. Quantification of lipid accumulation

Lipid accumulation was measured by Oil Red O extraction by lysis and gentle agitation 10 min at room temperature. We used 4 % NP-40 (Sigma-Aldrich, Prague, Czech Republic) in 100 % isopropanol as a lysis buffer. The extract (100 µl) was transferred into a 96 well plate. The absorbance was measured at 490 nm using a plate reader Synergy HT (BioTek, Winooski, VT, USA).

3. Indirect immunofluorescence

Cells were washed in PBS, fixed for 60 min in 4 % formaldehyde with PBS at room temperature, and permeabilized in PBS containing 0.3 % Triton X-100 for 15 min followed by blocking in PBS with 1 % bovine serum albumin (BSA) and 10 % normal donkey serum at room temperature for 60 min. After blocking, the cells were incubated with antibody against fatty acid binding protein 4 (FABP4; R&D Systems, Minneapolis, MN, USA) in working solution (PBS containing 0.03 % Triton X-100, 1 % BSA, 10 % normal donkey serum and anti-FABP4 in final concentration 10 µg/ml) overnight at 2-8 °C. After three 5-min rinsing in PBS with 1 % BSA, cells were incubated for 1 h in donkey anti-goat IgG NorthernLights™ NL557-conjugated secondary antibody (R&D Systems, Minneapolis, MN, USA) diluted 1:200 in 1 % BSA in PBS in the dark for 60 min at room temperature. The coverslips were washed, placed on microscope slides with a mounting medium (ProLong Gold Antifade Mountant with DAPI, Molecular Probes, Eugene, OR, USA) and visualized with a fluorescence microscope Olympus CX41 (Olympus, Tokyo, Japan) connected to a digital camera.

Qualitative and quantitative analysis of mitochondria

1. Fluorescent probe staining

Fluorescent probes, MitoTracker™ Red CMXRos and NucBlue® Live ReadyProbes® Reagent (both Molecular Probes, Eugene, OR, USA) were used to visualize mitochondria and nucleus. To label mitochondria, cells were incubated with MitoTracker™, which passively diffuses across the plasma membrane and accumulates in active mitochondria. Cell-permeant nuclear counterstain NucBlue® Live ReadyProbes® Reagent containing Hoechst® 33342 dye (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) emits blue fluorescence when bound to DNA with an emission maximum at 460 nm. It is detected through a blue/cyan filter.

Culture medium was replaced with Live Cell Imaging Solution (Molecular Probes, Eugene, OR, USA). Two drops of NucBlue® Live ReadyProbes® Reagent were added per milliliter of medium and MitoTracker™ was added in the final concentration 100 nM. Cells were incubated in the dark for 30 min and then visualized by the Hamamatsu Orca-ER camera mounted on the Olympus IX 81 inverted microscope at 200× magnification (Olympus, Tokyo, Japan).

2. High-resolution respirometry

Oxygen consumption by adipocytes was evaluated on days 0 (n=6), 4 (n=17) and 21 (n=18) of differentiation. To measure mitochondrial respiration of intact cells, oxygraph Oroboros O2k (Oroboros, Innsbruck, Austria) connected to the computer with DatLab software for data acquisition and analysis (Oroboros, Innsbruck, Austria) was used. The oxygen flux was calculated as a negative time derivative of the oxygen concentration in 2 ml glass chambers at 37 °C (Pesta and Gnaiger 2012).

StemPro® Adipogenesis Differentiation medium was stirred at 350 rpm and equilibrated with air for 60 min. After closing the chambers, the samples of intact adipocytes were injected into the chambers using Hamilton® syringe and respiratory activity of intact cells was assessed as routine respiration (ROUT; R). Then, non-phosphorylating LEAK state (L; oxygen consumption needed for electron transport compensating for proton leak across the inner mitochondrial membrane) was induced by addition of oligomycin (2.5 µmol/l), an ATP-synthase inhibitor. Maximum capacity of the electron transfer system (state E), was reached by titration of uncoupler trifluorocarbonylcyanide phenylhydrazone

(FCCP; 0.05 µmol/l titration steps). Oxygen consumption was then inhibited by inhibitor of complex III antimycin A (2.5 µmol/l) to achieve residual oxygen consumption (ROX). The cells were counted using Bürker´s hemocytometer (average cell count was ~4×10^5 cells per chamber) and oxygen consumption was expressed in pmol O_2/(s.10^6 cells) and corrected to ROX and instrumental background. The used protocol is shown in Figure 4A.

3. Citrate synthase activity

Determination of citrate synthase activity was used to estimate mitochondrial content in the samples from each oxygraph chamber. Two hundred microliters of the mixed and homogenized chamber content were added to 800 µl of the assay medium containing 0.1 mmol/l 5,5-dithio-bis-(2-nitrobenzoic) acid, 0.25 % Triton-X, 0.5 mmol/l oxaloacetate, 0.31 mmol/l acetyl coenzyme A, 5 µmol/l EDTA, 5 mmol/l triethanolamine hydrochloride, and 0.1 mol/l Tris-HCl, pH 8.1 (Kuznetsov *et al.* 2002). The enzyme activity was measured spectrophotometrically at 412 nm and 30 °C over 200 s and expressed in mIU per 10^6 cells

4. Mitochondrial membrane potential

The mitochondrial membrane potential was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Mitosciences, Abcam, Cambridge, UK). The cells were seeded at 1×10^4 cells on a dark well plate and cultured as explained earlier. The mitochondrial membrane potential was evaluated on days 0 (n=12), 4 (n=12), 10 (n=12) and 21 (n=12) of differentiation. The cells were washed once with PBS and incubated with JC-1 dye (1 µM) at 37 °C for 10 min. Then the cells were rinsed twice and were analyzed on a fluorescence spectrophotometer (Synergy HT, BioTek, Winooski, VT, USA) at excitation 475 nm and emission 530/590 nm. In addition, the changes in mitochondrial membrane potential were also analyzed by flow cytometer (FACSCanto II; BD Biosciences, San Jose, CA, USA). JC-1 solution (10 µl at the concentration of 200 µM) was added to cell suspension (1 ml) and cells were incubated at 37 °C for 25 min. Washing with PBS and centrifugation (1500 rpm; 5 min) followed the incubation period. The supernatant was removed; the pellet was resuspended in 500 µl PBS and immediately measured on the flow cytometer. The red/green fluorescence intensity ratio was determined to evaluate mitochondrial membrane potential.

Data analysis and statistics

Statistical analysis was performed using OriginPro 2017 software (OriginLab Corporation, Northampton, MA, USA). After testing for normality of distribution, data were compared using one-way ANOVA with Tukey's *post hoc* test. Values of $p<0.05$ were considered significant.

Results

Cell culture and differentiation

Human adipose-derived stem cells (hADMSCs) were maintained and cultured into differentiated adipocytes under standard conditions for 21 days. Cells harvested on day 0 were mesenchymal stem cells with typical fibroblastic spindle shape with several spurs and large nucleus. During adipogenic differentiation, cell morphology has changed – the cells gradually increased in volume, small fat vacuoles began to appear and then gradually increased in size (Fig. 1A-D). The phenotype of mature adipocytes was confirmed by the presence of FABP4 protein.

Cell proliferation and viability

As shown in Figure 2, a trend to increase the cell number was observed just after induction of differentiation (day 0 vs. day 4, $p=0.05004$) and it became significant thereafter (day 0 vs. day 10, $p=0.006$; day 0 vs. day 21, $p<0.0001$). In the later course of adipogenesis, mitotic division has slowed down (day 10 vs. day 21, $p>0.05$). This corresponds to our other results, where lipid accumulation is observed instead of mitotic division in the second phase of differentiation.

Cell viability determined by PrestoBlue™ reagent was expressed in fluorescent units (FU). No statistically significant changes in cell viability were observed during differentiation (Fig. 3).

Quantification of lipid accumulation

Oil Red O solution has been used to stain adipocytes and to visualize fat droplets. Oil Red O interacts with lipids to give a red-orange colored product. During differentiation, an insignificant increase in the fat content of the cells was observed and then, between days 10 and 21, fat accumulation significantly increased which corresponded to the increasing amount of measured Oil Red O staining (Figs 1A-D and 3C). The data are in accord with our previous finding that at this time, no significant mitotic division is taking place, but rather acquiring mature adipocyte-specific metabolic phenotype.

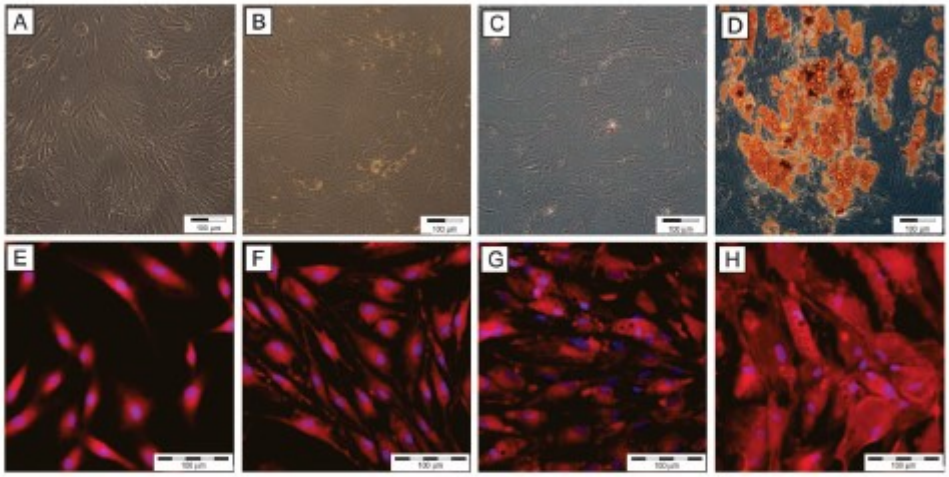

Fig. 1. Adipocyte differentiation (**A-D**) and mitochondria analysis using MitoTrackerTM (**E-H**). (**A**) Human adipose-derived mesenchymal stem cells stained with Oil Red O. (**B-D**) Adipocytes stained with Oil Red O on days 4, 10, and 21 of differentiation. (**E**) Mitochondria in human adipose-derived mesenchymal stem cells stained using MitoTrackerTM. (**F-H**) Mitochondria in adipocytes on days 4, 10, and 21 of differentiation stained by MitoTrackerTM.

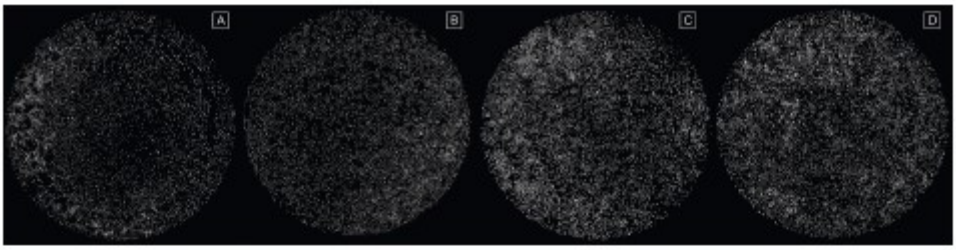

Fig. 2. Quantification of the number of cell nuclei (NucBlue®) in the course of adipocyte differentiation from human adipose-derived mesenchymal stem cells on days 0 (**A**), 4 (**B**), 10 (**C**), and 21 (**D**).

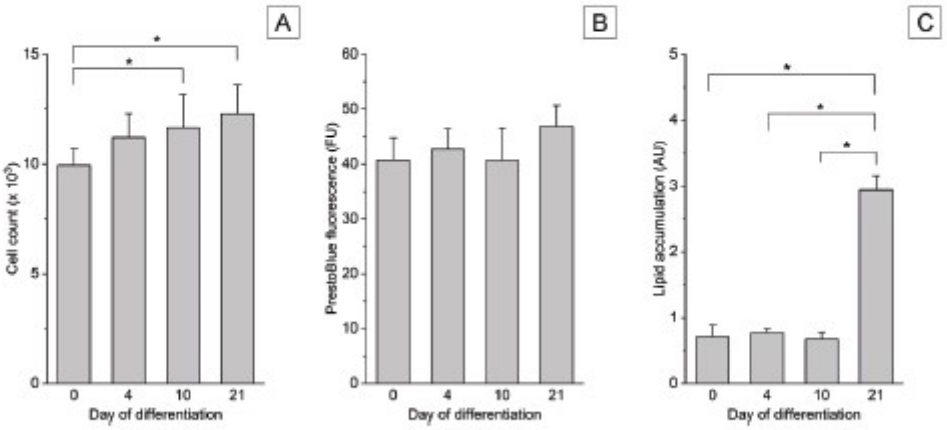

Fig. 3. (**A**) Proliferation (NucBlue®), (**B**) cell viability (PrestoBlueTM), and (**C**) lipid accumulation (Oil Red O) in adipocytes differentiating from human adipose-derived mesenchymal stem cells on days 0, 4, 10, and 21 of differentiation.

Qualitative and quantitative analysis of mitochondria

Fluorescence microscopy showed that during the adipocyte differentiation, the volume of mitochondrial networks apparently increased (Fig. 1E-H). These data corresponded with the results of determination of the citrate synthase activity. At the end of the differentiation, citrate synthase activity was 92.7±33.5 mIU/10^6 cells and it was significantly higher than on day 4 (17.9±4.5 mIU/10^6 cells) and day 0 (19.3±0.8 mIU/10^6 cells). The data suggest increasing mitochondrial quantity during differentiation.

High-resolution respirometry

As shown in Figure 4B-D, respirometric parameters have significantly changed in the course of adipogenesis. Basic respirometric states, i.e. R, L and E progressively increased in the course of differentiation (Fig. 4B). Interestingly, the most abrupt change of the state LEAK was observed between days 4 and 21. ATP-related oxygen consumption (R-L) and excess ETS capacity (E-R) increased significantly between days 0 and 4 and then they did not substantially change (Fig. 4C). LEAK control ratio, expressed as L/E was not different between days 0 and 4 of adipocyte differentiation and then it significantly increased on day 21. NetROUTINE control ratio expressing oxygen consumption related to ATP production (R-L) as a fraction of ETS capacity ((R-L)/E) increased between days 0 and 4 of the cell differentiation and then remained constant (Fig. 4D).

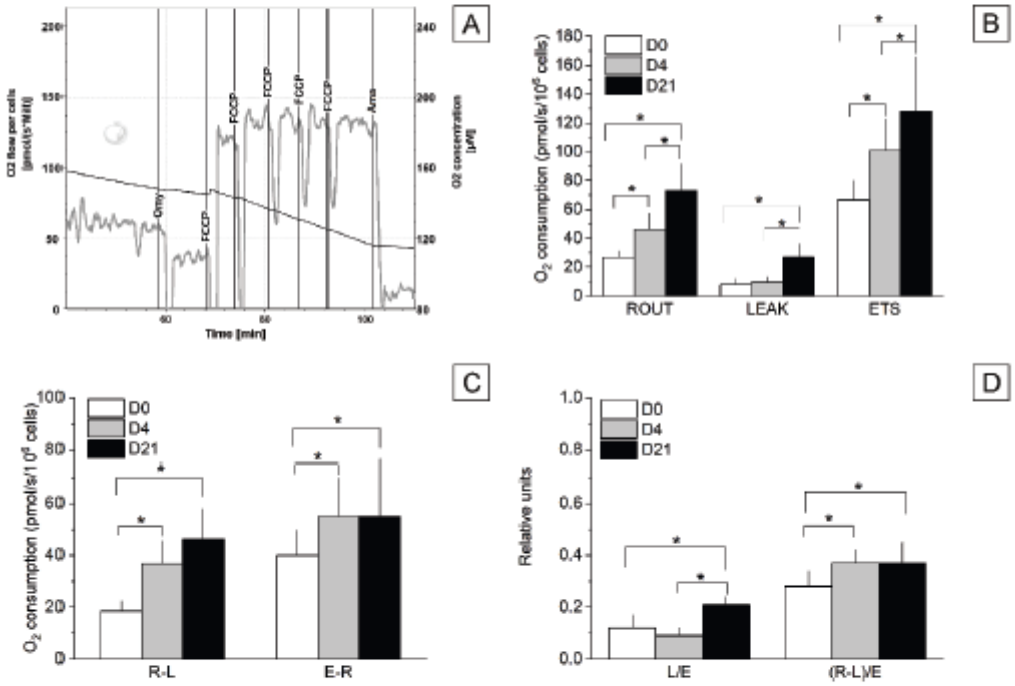

Fig. 4. Mitochondrial respiration in differentiating adipocytes on days 0, 10, and 21 of adipogenesis. (**A**) Titration protocol in adipocytes on day 21 of differentiation. Grey line=oxygen flux expressed in pmol/s per 10^6 cells. Black line=concentration of oxygen in the oxygraph chamber (µmol/l). Omy=oligomycin, FCCP=trifluorocarbonylcyanide phenylhydrazone, Ama=antimycin A. (**B**) Routine (R), LEAK (L), and uncoupled (E) oxygen consumption after addition of cells, Omy, and FCCP, respectively, after correction to residual oxygen consumption after Ama. (**C**) ATP-related oxygen consumption (R-L) and excess ETS capacity (E-R). (**D**) Flux control ratios documenting coupling state (L/E) and NetROUTINE control ratio ((R-L)/E). Significant differences $p<0.05$ (*).

Mitochondrial membrane potential

JC-1 dye is widely used to monitor mitochondrial health by measuring mitochondrial membrane potential. The low mitochondrial membrane potential is characterized by green fluorescence while high membrane potential is red. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. We observed

a decrease of this ratio during differentiation of hADMSCs to adipocytes by spectrophotometer assay (ratio 1.55 and 0.24 on days 4 and 21, respectively). The same trend was confirmed by flow cytometry (the ratio 3.19 on day 4 and 2.33 on day 21). The decrease of red fluorescence during differentiation is a signal of the mitochondrial depolarization and this trend fully corresponds to the results of high-resolution respirometry.

Discussion

The present study describes the increasing cellular oxygen consumption during 21-day adipogenic differentiation of human adipose-derived mesenchymal stem cells. It is well known that stem cells are highly glycolytic cells and have mechanisms that actively suppress mitochondrial respiration (Khacho and Slack 2017). Stem cells are probably less dependent on functional mitochondria for energy or metabolic needs but upon initiation of and during differentiation, a metabolic switch towards oxidative phosphorylation is necessary to get enough energy. This change must be logically accompanied by mitochondrial biogenesis. It appears that mitochondrial biogenesis and increased mitochondrial respiration is likely a general hallmark of adult stem cell differentiation (Hofmann *et al.* 2012, Zhang *et al.* 2013, Tang and Lane 2012).

Adipogenic differentiation of hADMSCs was induced using commercial culture medium and after four days, we observed very tiny lipid droplets. In differentiating cells, the number and size of lipid droplets increased the most abruptly between days 10 and 21 and this trend was documented by Oil Red O staining. The viability test quantifying reduction of the non-fluorescent resazurin to the fluorescent resorufin by the mitochondrial and cytoplasmic enzymes indicated that there was no trend to impaired cellular metabolism during adipocyte differentiation (Zalata *et al.* 1998).

All parameters associated with the potential ability of the cells to increase oxygen consumption, i.e. basic respirometric states, excess E-R capacity and ATP-related oxygen consumption (R-L) were significantly higher at the end of differentiation. Interestingly, the most abrupt changes of the state R-L and E-R were observed between days 0 and 4 and then they did not substantially change. This could be related to the fact that during the first four days of differentiation, the cells are able to divide and grow and their metabolic needs are increased. The amount of mitochondria increased the most between days 4 and 10/21 suggesting that the transition from glycolytic to oxidative metabolism energy is very fast but for the change of cell phenotype more time is required (Drehmer *et al.* 2016, Tang and Lane 2012).

Mitochondrial uncoupling during differentiation led to mitochondrial membrane depolarization and as a result, increased LEAK oxygen consumption needed for compensation for the proton leak. This would lead to substantial suppression of ATP generation (Bouillaud *et al.* 1984). However, in the cells undergoing adipogenesis, state ROUTINE continuously increased maintaining capacity to produce ATP sufficient for the metabolic needs, e.g. fatty acid synthesis (Zhang *et al.* 2013). Excess E-R capacity reflecting the maximum ability of mitochondria to enhance electron transport, increased only between days 0 and 4 of adipocyte differentiation and then it remained constant implying that after day 4, routine respiration could be increased only on the account of total electron-transporting capacity of mitochondria. High LEAK state and L/E uncoupling ratio on day 21 of adipogenesis could be related to increased expression of the uncoupling proteins UCP1, UCP2 and UCP3, which are responsible for disconnection between oxygen consumption and ATP production (Zhang *et al.* 2013). Another factor contributing to the increased LEAK state in the later stages of adipocyte differentiation could be activation of the mitochondrial permeability transition pore induced by long exposure to dexamethasone, regular component of media used for adipogenic differentiation (Chen *et al.* 2016, Luan *et al.* 2019).

In addition, parameters quantifying amount of mitochondrial mass fully corresponded with the above mentioned statements. The volume of mitochondrial networks and citrate synthase activity, marker of mitochondrial content (Larsen *et al.* 2012) increased during differentiation. Moreover, perinuclear localization of mitochondria was typical for undifferentiated cells, whereas mitochondria of mature adipocytes were more evenly distributed in the cytoplasm, especially around the lipid vacuoles. This observation was in accord with the study of Hofmann *et al.* (2012) and it supports the theory that the changes in distribution of mitochondria network could be a marker of successful differentiation of hADMSCs.

In summary, we showed that adipogenic differentiation of hADMSCs is a complex phenomenon

regulated on multiple levels and it is accompanied by changes of the mitochondrial phenotype. It suggests that mitochondrial metabolism should not be considered as a consequence of differentiation but a key mechanism in this process. Regulation of mitochondrial respiration may be an important therapeutic approach that could be explored as a new target to treat obesity-related disorders. Unlike the previous studies, our work provides an assessment of mitochondrial oxygen consumption by cells in the course of complete 21-day lasting adipogenesis and uses for the differentiation stem cells derived from human adipose tissue.

Study limitations

In this study, we have used human adipose-derived stem cells to determine basic mitochondrial characteristics in the course of their differentiation into mature adipocytes. Unlike commonly used human bone marrow mesenchymal cells (Charbord 2010) they enable less invasive recovery from patients while maintaining the same phenotype and functional characteristics. However, full adipogenesis is time-consuming and expensive process associated with many technical problems, like increasing fragility of the cells and their difficult harvesting. Although expression of FABP4 confirmed successful differentiation and maturation of adipocytes (Baxa *et al.* 1989), final cells did not contain a single lipid droplet typical for mature cells of white adipose tissue, but rather multiple lipid droplets resembling fat stores in the brite/beige adipocytes (Cedikova *et al.* 2016). In addition, mature cells loose viability early after the end of differentiation, which makes experiments with chronic exposure of these adipocytes to potentially harmful chemicals impossible. Nevertheless, they represent an excellent tool in research of adipogenesis from its very beginning (i.e. from the commitment of adipose-derived stem cells) in humans.

Conflict of Interest

There is no conflict of interest.

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Nové pohledy na význam rostlinných polyfenolů ve výživě

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Přehledová práce

Nové pohledy na význam rostlinných polyfenolů ve výživě

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Abstrakt

Rostlinné polyfenoly, přírodní látky přítomné ve všech potravinách rostlinného původu, jsou předmětem celosvětově rostoucího zájmu badatelů v oblasti lidské výživy. Zásadní nové poznatky vyplývající z tohoto výzkumu doplňují, případně mění dosud tradovaný obraz složitých metabolických drah těchto látek v lidském organismu, avšak stále ještě neumožňují odhalit spolehlivě takové jejich biologické účinky, jež by vysvětlovaly předpokládaný preventivní vztah těchto látek k neinfekčním chronickým chorobám. Na druhé straně nesporným závěrem výzkumu, zejména epidemiologických studií, je potvrzení, že zdravá výživa a zdravé potraviny by měly zahrnovat pravidelný a dostatečný příjem vhodných typů polyfenolů. Protože jejich průměrný celkový příjem je u české populace malý, je žádoucí stimulovat u všech populačních skupin, zejména rizikových, zájem o potraviny bohaté na biologicky nejúčinnější podskupiny rostlinných polyfenolů, případně nabízet je v doplňcích potravy.

Úvod

Pozornost, která je v odborné veřejnosti věnována rostlinným polyfenolům, v posledním desetiletí exponenciálně stoupá. Svědčí o tom počet vědeckých publikací zaměřených na různé aspekty těchto přirozených látek. Zatímco do roku 1990 počet článků, věnovaných nejdříve chemickým strukturám a později metodám stanovení, úloze polyfenolů v senzorické kvalitě potravin a posléze jejich biologickým účinkům a dalšímu rozšiřování poznatků o jejich výskytu a o velikosti jejich příjmu, nepřekročil v úhrnu jednu tisícovku, v období následujícím až doposud je jich evidováno přes 90 tisíc [1]. Žádné jiné skupině přirozených látek v rostlinném materiálu není věnována podobná pozornost. Celkový počet vědeckých prací o flavonoidech např. převyšuje počet prací o vitaminech C a E, o karotenoidech a o selenu dohromady. Mění se také předmět studia a cíl vědeckého bádání a jeho výstupů; pro uplynulých 10 let je příznačné, že se pozornost soustřeďuje na látkovou přeměnu polyfenolů v lidském organismu, na povahu biologicky aktivních metabolitů, na důkaz existence a podmíněnosti jejich vlivu na zdraví a na hledání a ověřování možností využití potravin (popř. potravin doplněných polyfenoly) k upevňování zdraví a k účinné prevenci nemocí [2]. Pro tento obor nutričního výzkumu je typické, že důležitými prameny poznatků už nejsou jen výsledky epidemiologických studií (kterých je nepřehledně mnoho), ale jejich metaanalýzy a metaanalýzy metaanalýz. Svou roli ostatně také hraje skutečnost, že rostlinné polyfenoly jsou nejpočetnější skupinou fytochemických látek (dosud bylo identifikováno přes 9 tisíc chemických individuí) a obsah polyfenolů převyšuje obsah vitaminů a esenciálních mikronutrientů. Jejich celkový denní příjem převyšuje u všežravých dospělých osob 1 g, u lidí vegetariánsky orientovaných a konzumujících větší dávky kávy a čaje, kakaových výrobků, piva a vína může dosahovat 3 a více g/den [3]. Téměř všechny tyto látky vykazují samy o sobě (a také potraviny, které je obsahují) v testech vysokou antioxidační aktivitu. Už starší zkušenosti ukazovaly, že s větším a pravidelným konzumem ovoce, zeleniny, luštěnin, výrobků z obilovin, čaje, kávy a vína se významně posiluje ochrana zdraví před neinfekčními degenerativními chorobami (kardiovaskulárními, nádorovými, neurodegenerativními, diabetem 2. typu aj.) a také naděje na dožití ve vyšším věku a v dobrém zdraví. Dnes je středem pozornosti příčina zdravotní prospěšnosti polyfenolů a mechanismus jejich účinku, který není zdaleka podmíněn pouze jejich antioxidačním charakterem [4].

V tomto sdělení stručně shrneme závěry, ke kterým studium různých aspektů výskytu polyfenolů v potravinách, různých stránek jejich biologické aktivity a prokazatelných účinků na zdraví lidí v období posledních přibližně 10 let dospělo. Naším cílem je také upozornit na omyly, které se v laické veřejnosti ohledně fenolových fytochemických látek vyskytují.

Současný stav znalostí o obsahu rostlinných polyfenolů v potravinách

Metodické problémy se stanovením obsahu polyfenolů v potravinách už byly uspokojivě vyřešeny. Velmi oblíbená, ale nárokům na specifičnost a spolehlivost nevyhovující je metoda založená na reakci fenolové skupiny s molybdenanem v alkalickém roztoku (tzv. Folin-Ciocalteuova metoda), která se používá pouze k předběžnému a orientačnímu stanovení. Moderní metody jsou založeny na citlivých dělicích postupech s využitím vysokoúčinné kapalinové chromatografie, která je případně doplněna hmotnostní spektrometrickou identifikací. Od roku 2009 je k dispozici online databáze, obsahující hodnoty obsahu polyfenolů v potravinách, které byly staženy z 1 300 odbor-

Tabulka č. 1.

Příklad rostlinných zdrojů s nejvyššími obsahy polyfenolů

Potravina	Obsah polyfenolů	Typ polyfenolu
Kapary	655 mg/100 g	převažují flavonoly
Máta peprná	1490 mg/100 g	flavony
Sójová mouka odtučněná	477 mg/100 g	isoflavony
Bezinky	1316 mg/100 g	antokyanidiny
Černý rybíz	592 mg/100 g	antokyanidiny
Kakaový prášek	512 mg/100 g	flavanoly
Kaštany jedlé	1215 mg/100 g	kyselina benzoová
Káva rozpustná	330 mg/1 šálek	kyselina chlorogenová

Tabulka č. 2.

Žebříček velikosti denního příjmu polyfenolů

Stát	Denní příjem polyfenolů
Dánsko	1706 mg
Francie	1193 mg
Polsko	1092 mg
Finsko	863 mg
Španělsko	826 mg
Řecko	664 mg
Brazílie	460 mg
Česká republika	426 mg

ných článků. Jinou velmi obsáhlou a stále široce využívanou databázi uveřejnili v roce 2010 Neveu a spol. [5]. Hodnoty obsahů polyfenolů v databázi byly získány laboratorními analýzami minimálně 10 vzorků každého druhu potraviny (získaných z různých světových oblastí) a byly použity paralelně čtyři různé analytické metody - spektrofotometrická s Folin-Ciocalteuovým činidlem a tři různé modifikace vysoce účinné kapalinové chromatografie (HPLC). Je nutné ale respektovat skutečnost, že ani tak důkladným přístupem se nemohou získat univerzálně použitelné výsledky, protože obsah polyfenolů v každé potravině rostlinného původu se může velmi podstatně lišit v závislosti na odrůdě, na klimatických podmínkách pěstování, na agrotechnických podmínkách a na mnoha dalších okolnostech. Uvádíme příklady rostlinných zdrojů s nejvyššími obsahy polyfenolů, viz Tabulka č. 1.

Na základě znalostí velikosti spotřeby jednotlivých druhů potravin se vypočítává průměrný denní příjem polyfenolů na 1 osobu. Tento údaj je v epidemiologických studiích a při každém jiném způsobu hodnocení vlivu potravních polyfenolů na zdraví velmi důležitý, avšak jeho validita je často zpochybňována, protože dosud nebyly vypracovány zcela spolehlivé individuální dotazníky o spotřebě potravin a databáze obsahů polyfenolů v potravinách je možné používat jen s vědomím jejich přibližnosti.

Z přehledu relevantních epidemiologických studií vyplývá tento žebříček velikosti denního příjmu polyfenolů, viz Tabulka č. 2.

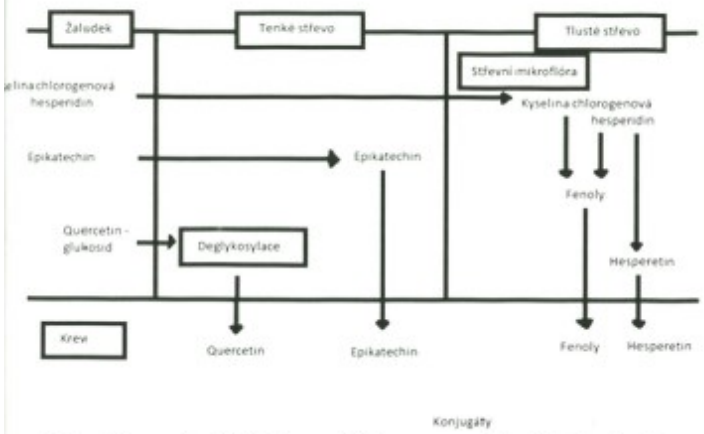
Hlavními zdroji polyfenolů v naprosté většině zemí, kde byly hodnoceny, byla káva, čaj, ovoce (v pořadí bobulové, jádrové a citrusové) a kakao, v České republice také obiloviny a pivo. Z jednotlivých skupin fenolových látek v Evropě převažují flavanoly (káva, kakao, čaj, jablka), flavanony (citrusy), kyseliny hydroxyskořicové (káva, bobulové ovoce), flavonoly (cibule, jablka, čaj) a antokyanidiny (barevné ovoce). V České republice se podle naší studie uplatňují nejvíce hydroxyskořicové kyseliny (z 30 %) a dále flavony, flavanoly a antokyanidiny (dohromady ze 42 %) [6]. Ve všech úvahách o velikosti a struktuře příjmu rostlinných polyfenolů se dosud vědomě opomíjí užívání polyfenolových doplňků potravy a funkčních potravin s dominantním obsahem těchto fytochemických látek. Není také podle našeho názoru dosud vědecky zhodnocen skutečný dopad těchto přípravků na zdraví lidí.

Biologická přístupnost a metabolismus polyfenolů zásadním způsobem limitují jejich biologickou aktivitu

Zcela zásadní otázkou zdravotní prospěšnosti rostlinných polyfenolů je míra a povaha jejich využití v lidském organismu. Polyfenoly jsou ve svých rostlinných zdrojích uloženy ve vnitrobuněčném (často ve vakuolách) i v mimobuněčném prostředí. Vždy jsou doprovázeny velkým množstvím organických látek, často makromolekulární povahy, s nimiž jsou navázány vazbami. Charakter této tzv. matrice je rozhodující pro míru rozpustnosti, chemického štěpení a velikosti vstřebání polyfenolů v trávicím ústrojí. Tím je také podmíněna velikost a povaha jejich biologického účinku. Základem matrice jsou různé polysacharidy nebo heteropolysacharidy, bílkoviny a tuky, často se jedná o makromolekuly, které se zahrnují do kategorie vlákniny potravy (polymerní polyfenoly, např. proantokyaniny nebo polymerní katechiny se přímo s vlákninou ztotožňují). V odborné literatuře se tak pojednává o potenciálu „bioaccessibility", což zahrnuje především způsobilost polyfenolů být předmětem trávení, a „bioavailability", která se týká procesu vstřebání, metabolismu a transportu polyfenolů a jejich štěpných produktů k cílovým tkáním [7]. Na tyto aspekty je v poslední době kladen velký důraz a jsou podrobovány důkladnému studiu, neboť jen jejich znalost otevírá cestu k dalšímu ověřování skutečné biologické aktivity polyfenolů a jejich ovlivňování zdraví [8]. V této oblasti výzkumu bylo v posledním desetiletí shromážděno nejvíce cenných, často překvapujících poznatků.

Využitelnost polyfenolů přítomných v rostlinné potravě je podmíněna nejen povahou matrice, na kterou jsou v potravě vázány, ale také způsobem kulinářské úpravy, zejména tepelnými zákroky, které mohou trávení polyfenolů usnadnit, nebo naopak snížit nebo zcela znemožnit. Trávení polyfenolů může probíhat už v žaludku (žaludečními amylasami) a v největší míře v celé délce tenkého střeva. Zde se účinkem pankreatických a intestinálních amylas a glykosidas štěpí především vazby mezi aglykony a sacharidy (glykosidy jsou prakticky výhradní formou jednoduchých polyfenolů v potravě).

Obrázek 1.
Metabolická cesta příjmu, absorpce a metabolismu polyfenolů [13]



Toto schéma nově zohledňuje a osvětluje procesy probíhající v tlustém střevu.

zjištěno, že polyfenoly se mohou vázat na bílkoviny trávicích enzymů a tím jednak inhibovat jejich aktivitu a jednak snižovat svou antioxidační účinnost. Na druhé straně se určitá část polyfenolů vstřebá v původní nebo málo pozměněné podobě. Tyto zbytky jsou v játrech podrobeny biotransformacím 1. typu (hydroxylace, oxygenace aj.) a následně biotransformacím 2. typu, zejména methylaci a O-methylaci, sulfataci a glykosidické vazbě s kyselinou D-glukuronovou. Uronidglukosidy, sulfáty a methylderiváty polyfenolů a jejich fragmentů jsou běžně identifikovány v různých tkáních a v moči, ovšem obvykle v menší než mikromolární koncentraci. Polyfenoly jsou tak pravděpodobně zčásti v těle katabolizovány jako xenobiotika, látky tělu cizí (týká se benzenových cyklů). Zásadním

V posledních letech bylo opakovaně prokázáno, že toto štěpení a také rozklad oligomerních a polymerních forem polyfenolů (taniny, leukoantokyanidiny) probíhají v tenkém střevu člověka jen v omezené míře nebo neprobíhají vůbec a nestrávené zbytky jsou přenášeny do tlustého střeva. Zde se stávají výživovým substrátem pro velké počty a početné druhy bakterií, které napadají různé části molekul polyfenolů včetně benzenových jader. Potvrzuje se, že i tyto metabolity se mohou stěnou tlustého střeva vstřebat a vstupovat do systémového oběhu, část z nich (mnohdy podstatná) se z těla vyloučí. V přítomné době se realizuje mnoho studií, které in vitro kombinují různé podmínky simulující prostředí trávicího ústrojí a pátrají po osudu polyfenolových struktur. Příkladem jsou modely obsahující kyselinu chlorovodíkovou, pepsin nebo mírně zásadité prostředí a žluč. Oblíbená je aplikace kultur nádorových buněk ze stěny tlustého střeva, pomocí nichž se studuje průběh absorpce polyfenolů a produktů jejich trávení. Bylo tak např. zjištěno, že jablečné polyfenoly se u člověka štěpí na aglykony a sacharidy už v žaludku, a to z 65 %, zbytek se rozkládá v tenkém střevu. Po aplikaci antokyanidinů značkovaných radioisotopem ^{14}C byly nalezeny značkované metabolity (typu acetyl- nebo valerylfenolů a fenyloctové kyseliny aj.) v krvi, v moči a ve stolici pokusných osob a také v jejich dechu (12 % ^{14}C z původní dávky), což naznačuje hluboký strukturní rozklad polyfenolů ve střevu a jejich velký podíl, který nebyl v organismu využit. Velká část přírodních fenolových látek přijímaných potravou přechází až v 90 % podílu do tlustého střeva. Koncentrace polyfenolů a jejich metabolitů vzniklých v tenkém střevu a ihned vstřebaných dosahuje po nasycení v krvi hodnoty přibližně 1 μmolu na litr, zatímco koncentrace metabolitů vzniklých v tlustém střevu bývá v krvi 10 až 100 krát větší. Bylo také opakovaně

a poměrně novým poznatkem tak je skutečnost, že reálné využití rostlinných polyfenolů v těle člověka je velmi omezené a je velmi závislé na matrici, na kterou je vázáno, na způsobu tepelné úpravy potravy a zejména na složení střevní bakteriální mikrobioty [9, 10]. Stručné schéma znázorňující hlavní metabolické dráhy polyfenolů v lidském organismu je znázorněno na obr. 1.

Biologická aktivita polyfenolů je velmi vágní a její poznání je možné jen pomocí klinických studií

O polyfenolech se již po dlouhou dobu soudí, že mají příznivý účinek na zdraví. Tento předpoklad je z dnešního hlediska oprávněný pravděpodobně ve smyslu posilování prevence některých chorob. Jestliže větší spotřeba ovoce, zeleniny, obilovin, luštěnin, popřípadě také čaje, kávy a kakaových produktů jsou asociovány s nižší incidencí neinfekčních chronických nemocí, považuje se za důvodné, že důležitým příznivým faktorem je dostatečný příjem fytochemických látek typu rostlinných polyfenolů. V přítomné době se volá po rozšíření klinického výzkumu těchto látek (dosud se vychází převážně z výsledků epidemiologických studií a experimentů s pokusnými zvířaty), které by umožnily zdokonalit poznání příčinných souvislostí a mechanismů biologických účinků polyfenolů. Přetrvávajícím nedostatkem je převažující zájem výzkumníků o flavonoidy, které tvoří jen část širokého spektra polyfenolů v naší potravě. Ve skutečnosti více než polovinu polyfenolů představují v evropské populaci deriváty kyseliny benzoové a hydroxyskořicové. V posledních několika letech byly publikovány výsledky studií na buněčných a tkáňových kulturách a pokusů s lidskými dobrovolníky, jejichž výsledky dovolují formulovat hypotézy o povaze biologických účinků polyfenolů a jejich metabolitů na molekulárně chemické úrovni [11].

Tyto práce se zaměřují na důležité a perspektivní stránky problematiky účinků polyfenolů na zdraví: jedná se o jejich potenciální účast v pochodech karcinogeneze, respektive buněčné proliferace a tkáňové diferenciace a programované smrti, ovlivnění sekrece a účinků inzulínu a vliv na postprandiální glykémii, vliv na apetit člověka a na jeho energetický metabolismus ve vztahu k udržení nebo úpravě tělesné hmotnosti, vliv na tukový metabolismus (včetně zvýšeného obratu cholesterolu a inhibice lipas), vliv na snížení napětí v cévách a na krevní tlak a vliv na neurodegenerativní procesy. V souvislosti s tím se stále věnuje pozornost, i když v omezenější míře, antioxidační aktivitě polyfenolů ve smyslu žádoucí, ochranné funkce před škodlivými účinky reaktivních forem kyslíku a dusíku (ROS, NOS). Přehled dosud získaných převážně dílčích výsledků je velmi široký a při značném zjednodušení jej lze shrnout konstatováním, že některé polyfenoly (zejména flavanoly/katechiny, flavonoly, resveratrol, antokyaniny) pravděpodobně za určitých podmínek stimulují expresi genů kódujících antioxidační enzymy a cytoprotektivní proteiny; mechanismus této stimulace je založen na podpoře fosforylace serinových skupin na oxidačně redukční regulaci cysteinových skupin aktivních bílkovin a peptidů (enzymových, transkripčních faktorů, cytokinů aj.) a v důsledku toho na modulaci buněčných signálních přenosů [12].

Velmi nesnadným, ale důležitým úkolem je vymezení těch druhů polyfenolových sloučenin, u kterých se taková biologická aktivita předpokládá a na něž by se zaměřil další intenzívní výzkum.

V přítomné době se za zdravotně velmi prospěšné považují přírodní látky přítomné v čaji zeleném, černém i v jeho ostatních variantách, v kávě (není-li kontraindikována, doporučuje se 3 a více šálků denně), v kakaových výrobcích, v bobulovém ovoci, zejména v borůvkách, hroznech (červených a také v červeném víně), dále v jablkách, citrusech, luštěninách, zázvoru a v odtučněné sójové mouce.

Je účelné užívání funkčních potravin obohacených polyfenoly nebo polyfenolových doplňků?

Výběr potravin, které jsou zdrojem „zdravých" polyfenolů, je poměrně snadný. Naše obchody disponují širokým sortimentem potravin, které nemusí být pouhým prostředkem k nasycení, ale mohou pozitivně ovlivňovat zdraví a jsou pro velkou část populace cenově dostupné. Jedná se především o potraviny rostlinného původu.

Přesto jsme zaznamenali v ČR podprůměrný příjem rostlinných polyfenolů [6]. V zájmu podpory veřejného zdraví je proto žádoucí stimulovat větší zájem o zdroje polyfenolů, ale nabízí se také možnost užívání speciálních doplňků potravy a přípravy funkčních potravin obohacených těmito přírodními látkami. Tato praxe je ve světě dnes běžná, globální trh s polyfenolovými preparáty získává téměř 1 miliardu US dolarů ročně a nepřetržitě roste. V západní Evropě a v USA se nejvíce prodávají extrakty z bohatých zdrojů, jako je zelený čaj nebo odtučněná sójová mouka.

Cílové skupiny, k nimž by měla nabídka polyfenolových zdrojů směřovat, jsou lidé ohrožení chronickým neinfekčním onemocněním, jako jsou srdečně cévní, nádorové a neurodegenerativní nemoci a diabetes 2. typu, a všeobecně všichni senioři. Je možné vycházet ze skutečnosti, že polyfenoly jsou málo toxické, mnohdy ale mají zhoršené senzorické a fyzikálně chemické vlastnosti, což lze kompenzovat ochucením, emulgací apod. úpravami. Protože mají velmi rychlý metabolický obrat, měly by se konzumovat pravidelně v krátkých časových intervalech, pokud možno společně s běžnou potravou. Za zmínku stojí skutečnost, že velikost příjmu potravy rostlinného původu a s ním také rostlinných polyfenolů lze kontrolovat a testovat pomocí jednoduché semikvantitativní analýzy moči. Na našem pracovišti jsme úspěšně vyzkoušeli rychlý a jednoduchý test na celkový obsah polyfenolů a jejich metabolitů založený reakcí s Folin-Ciocalteuovým

činidlem (test činidlem na fenolové látky), na obsah kyseliny askorbové (titračně s 2,6-dichlorfenolindofenolem, indikuje velikost konzumu ovoce a zeleniny)a na celkovou antioxidační kapacitu (např. metodou FRAP s železitou solí a ferrikyanidem draselným, odráží přítomnost dalších antioxidantů).

Závěr

Velký počet epidemiologických studií uskutečněných v posledním desetiletí prokazuje nesporné zdravotně prospěšné účinky rostlinných polyfenolů, které se vyskytují všudypřítomně v potravinách rostlinného původu. Platnost tohoto zjištění neodporuje skutečnosti, že polyfenoly jsou v lidském organismu využity jen z malé části a jejich metabolismus je podstatně ovlivněn bakteriálním osídlením v tlustém střevu, které je interindividuálně značně rozdílné. Navíc struktura fenolových metabolitů, které jsou biologicky aktivní, ani molekulárně chemický mechanismus jejich účinku nebyl dosud spolehlivě objasněn. Považuje se za velmi pravděpodobné, že biologicky nejaktivnější typy polyfenolů jsou flavany, flavanoly (katechiny) a flavonoly, jejichž hlavními potravními zdroji jsou čaj zelený i černý, káva, kakao, červené hrozny a víno a intenzívně barevné bobulové ovoce. V české populaci je příjem těchto látek nedostatečný. Zlepšení situace lze dosáhnout změnou spotřebního koše potravin (ve prospěch zdrojů flavonoidů) a nabídkou polyfenolových doplňků potravin.

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Abstract

Plant polyphenols are natural substances present in foods of plant origin. They are the subject of worldwide growing interest in human nutrition reserchers. The fundamental new knowledge it follow that this research complement or change the imagine of the metabolit patheways of these substances in the human body, but still do not allow them to reliably detect their biological effects that would explain their predicted relationship to non-infectious chronic diseases. On the other side, the indisputable conclusion of research, especially epidemiological studies, is the confirmation that the healthy nutrition and healthy food should include regular and sufficient intake of suitable types of polyphenols. Because polyphenols average total intake for Czech population is small, it is desirable to stimulate interest in food rich in the most biological effective supgroups of plant polyphenols or offer them in food supplements for all population groups, especially vulnerable group.