Modulation of insulin-induced genotoxicity in vitro and genomic damage in gestational diabetes

Modulation der Insulin-induzierten Genotoxizität in vitro und Genomschäden bei Frauen mit Gestationsdiabetes

Dissertation for Submission to a Doctoral Degree
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Abbreviations

°C	Degree Celsius
HO•	Hydroxyl radical
4E-BP1	Eukaryotic initiation factor 4E (eIF-4E)-binding protein 1
8-OxodG	8-Oxo-2'-deoxyguanosine
AKT	Serine/threonine protein kinase
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CBPI	Cytokinesis block proliferation index
DABCO	Diazabicyclo octane
DAPI	4,6-Diamidino-2-phenylindole
DDR	DNA damage response
DHE	Dihydroethidium
DM	Diabetes mellitus
DMEM	Dulbecco modified Eagle's minimal essential medium
DN	Double nucleated
DNA	Deoxyribonucleic acid
DSB	Double-strand break
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDTA	Ethylenediamine-tetraacetic acid disodium salt
ER	Estradiol receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FRAP	Ferric reduction antioxidant power
GDM	Gestational diabetes mellitus
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
GSH	Reduced form of glutathione
h	Hour

H_2O_2	Hydrogen peroxide
HL-60	Human promyelocytic leukemia cell line
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HRP	Horse radish peroxidase
IR	Insulin receptor
IRS 1/2	Insulin receptor substrate
Kg	Kilogram
LMP	Low-melting-point agarose
M	Mole per liter
mA	Milliampere
MAPK	Mitogen-activated protein kinase
min	Minute
MN	Micronucleus
mRNA	Messenger ribonucleic acid.
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids (NEAA
NQO	4-Nitroquinoline 1-oxide
NRK	Normal rat kidney epithelial cell line
PBMC's	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pH	Potential of hydrogen
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PIC	Protease inhibitor cocktail
PIP2	Phosphatidylinositol-3,4,5-biphosphate
PIP3	Phosphatidylinositol,-3,4,5-triphosphate
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
Rheb	Ras homolog enriched in brain
ROS	Reactive oxygen species
rpm	Revolutions per minute

SSB	Single-strand break
S6K1	Ribosomal protein S6 kinase1
SCGE	Single cell gel electrophoresis
SD	Standard derivation
SH2	Src homology 2
SOD	Superoxide dismutase enzyme
T1DM	Diabetes mellitus type 1
T2DM	Diabetes mellitus type 2
T3	Triiodothyronine
T4	Thyroxine
Tris	Tris(hydroxymethyl)aminomethane
TRs	Thyroid hormone receptors
TSC-2	Tuberous sclerosis complex 2
UV	Ultra violet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WHO	The World Health Organization

1 Introduction

1.1 DNA damage

Genomic damage is usually caused when DNA is exposed to physical and chemical mutagens such as ultraviolet radiation (200-300 nm wavelength) and ionizing radiation (X-rays), hydrolysis or thermal disruption and endogenous reactive oxygen species (ROS) that accumulate due to metabolic processes in cells. DNA damage occurs at a frequency of tens of thousands of DNA lesions per day for each of the $\sim 10^{13}$ cells in human body [1]. However, only a small part of ca. 6 billion bases or unrepaired lesions in critical genes (e.g. tumor suppressor genes) can interfere with cellular functions and increase the ability of tumor formation [2]. Mutations can be one of the consequences of DNA lesions interfering with essential cellular processes, such as transcription or replication which plays a major role in the development of cancer and aging [3].

Different DNA damage types have been recognized and could be classified into four main groups as illustrated in Figure 1 [4]:

a) DNA adducts and single-strand breaks which are created via oxidation, alkylation and hydrolysis of DNA bases.

b) Bulky adducts and pyrimidine dimers (intrastrand cross-links) which are formed due to various chemicals and UV irradiation.

c) Double DNA strand breaks, and interstrand cross-links that are produced by ionization radiation, oxidizing agents, chemicals, inhibition of DNA polymerase and topoisomerase and other sources.

d) DNA mismatches during DNA replication by wrongly adding or removing DNA bases to the new DNA strand or inhibiting the replication enzymes.

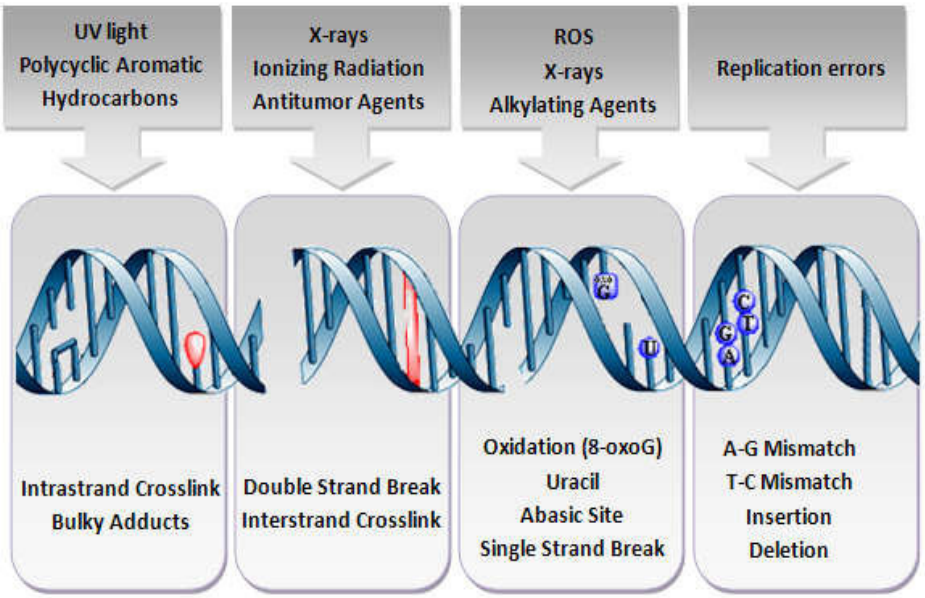


Figure 1. The major DNA damage lesions, including double-strand break (DSB), single-strand break (SSB), DNA adducts and cross-links formation, and base mismatch, can be induced by different exogenous and endogenous DNA damaging factors. Modified from Boland et al. [4].

1.1.1 **DNA damage response**

The DNA damage response (DDR) is a network of DNA repair processes to protect the cells by removing different types of DNA lesions regardless of the type of DNA damage [5, 6]. The response pathway starts after it senses the DNA damage and replication stress, followed by a set of protein kinase transducers to mediate the repair processes [7]. Different signaling cascades involved in DDR mediate various cellular responses (effectors) including the arrest of the cell cycle for repair of the damage before replication or mitosis [8, 9], or induction of apoptosis when the DNA damage is irreparable [10, 11] (Figure 2).

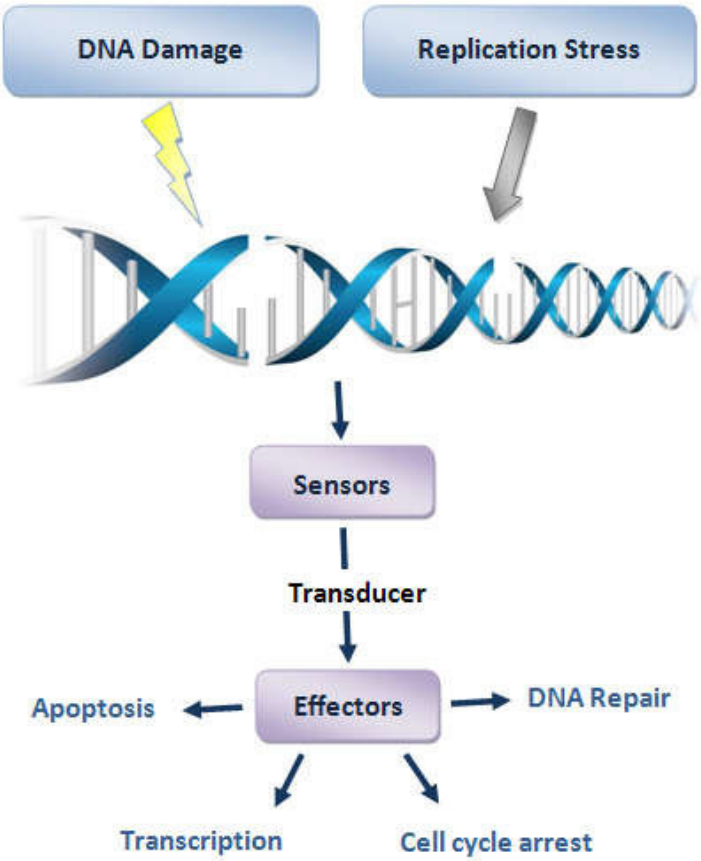


Figure 2. General outline of the signal-transduction pathway of the DNA damage response (DDR) including activation of checkpoints for cell cycle arrest, transcriptional programs, DNA repair and apoptosis. Modified from Zhou and Elledge [9].

1.2 Reactive oxygen species (ROS)

During normal cellular metabolism in mammalian cells, reactive ions or small molecules including oxygen ions, free radicals and peroxides are continuously generated [12]. The free radicals can be formed by losing or gaining an electron or breaking of a covalent bond to create one or more unpaired electrons [13]. Among these molecules, the most important are $O_2^{\bullet -}$, H_2O_2, and $HO^{\bullet}$ which are known as reactive oxygen species (ROS) [14].

ROS are involved in several biological functions such as the regulation of cell growth and differentiation, modulation of extracellular matrix, stimulation of gene expression and they serve as second messengers in signal transductions. On the other hand, high

levels of ROS play a significant role in DNA damage induction that can affect functions of cells [15]. Their effects on DNA range from formation of different oxidative DNA adducts (base modifications), single- and double-strand breaks, deoxyribose oxidation, and cross-links of oxidized bases to single and double-strand breaks [16, 17]. The most common adduct is 8-oxodeoxyguanine (8-oxodG) that is formed due to the addition of $HO^{\bullet}$ to guanine (G) at C-8. Thus, 8-oxodG has been considered as an important oxidative damage biomarker [18, 19]. The main cellular endogenous sources are well described regarding ROS production. Among them are the NADPH oxidase enzyme family and the mitochondrial respiratory chain due to their high oxygen consumption as well as other sources e.g. endoplasmic reticulum and peroxisomes [20]. However, production of $O_2^{\bullet -}$ can be also elevated by exogenous sources like pollution, smoking, pesticides and radiation [20].

The NADPH oxidase enzyme complexes are transmembrane proteins and generate $O_2^{\bullet -}$ and H_2O_2 by catalyzing the electrons transfer across the plasma membrane from NADPH to reduce the oxygen molecule [21, 22]. NADPH oxidases are found in seven isoforms, NOX1 to NOX5 and Duox1 and Duox2 with different catalytically active subunits [23]. Based on the level of expression, it can be concluded that the NOX homologues contribute differently to many biological and pathological functions [21, 23]. Additionally, NOX isoforms generate different types of ROS. For example, superoxide $O_2^{\bullet -}$ formation depends on Nox1 and Nox2 [24, 25], while Nox4 is responsible for the basal H_2O_2 generation [25, 26], and Nox5 releases H_2O_2 dependent on Ca^{2+} [27].

Moreover, mitochondria play an essential role in the cellular survival and the production of energy/ATP. The mitochondrial respiratory transport chains (ETC), which are composed of four complexes (I–IV) with ATPase as complex V are responsible for ATP production via the tricarboxylic acid cycle (TCA cycle), electron transfer and oxidative phosphorylation (OXPHOS) [28]. Additionally, reactive oxygen species (ROS) are generated by ETC and this activity has been related to the development of aging, diabetes and pathogenesis of neurodegenerative diseases [20, 29].

The mitochondrial oxidative phosphorylation is the major source for energy production through combination of the electron transport chain with cellular respiration and ATP synthesis. However, the oxidative phosphorylation is based on the transfer of negatively charged electrons through the mitochondrial respiratory chain via redox reactions towards accepter components with more positive reduction potentials until they finally reduce oxygen molecules to water.

Complex I (NADH dehydrogenase) passes the electrons from the electron rich molecule NADH to coenzyme Q (ubiquinone). While electrons can be donated by succinate at complex II (succinate dehydrogenase, SDH) to coenzyme Q and transferred to complex III (ubiquinol cytochrome c reductase) which mediates the electron transfer chain to pass electrons from reduced coenzyme Q to cytochrome c [30, 31]. Complex IV (cytochrome c oxidase) uses electrons from cytochrome c to reduce oxygen molecules to water and pump protons (H^+) from the mitochondrial matrix across the intramembrane which produces an internal negative charge and a pH gradient [31] (Figure 3).

The proton gradient generates a proton motive force, which is utilized by ATPase-complex V to phosphorylate ADP and release ATP [32]. Under physiological conditions, around 90% of the total cellular ROS is generated by mitochondrial ETC [28]. Due to the leakage of some electrons through the mitochondrial ETC, particularly from complexes I and III, about 0.2–2% of oxygen consumed is reduced to superoxide [33]. The mitochondrial superoxide is converted to H_2O_2 by antioxidant mechanisms to produce $OH^{\bullet}$ (primary radical). The main antioxidant mechanisms are enzymatic defense systems.

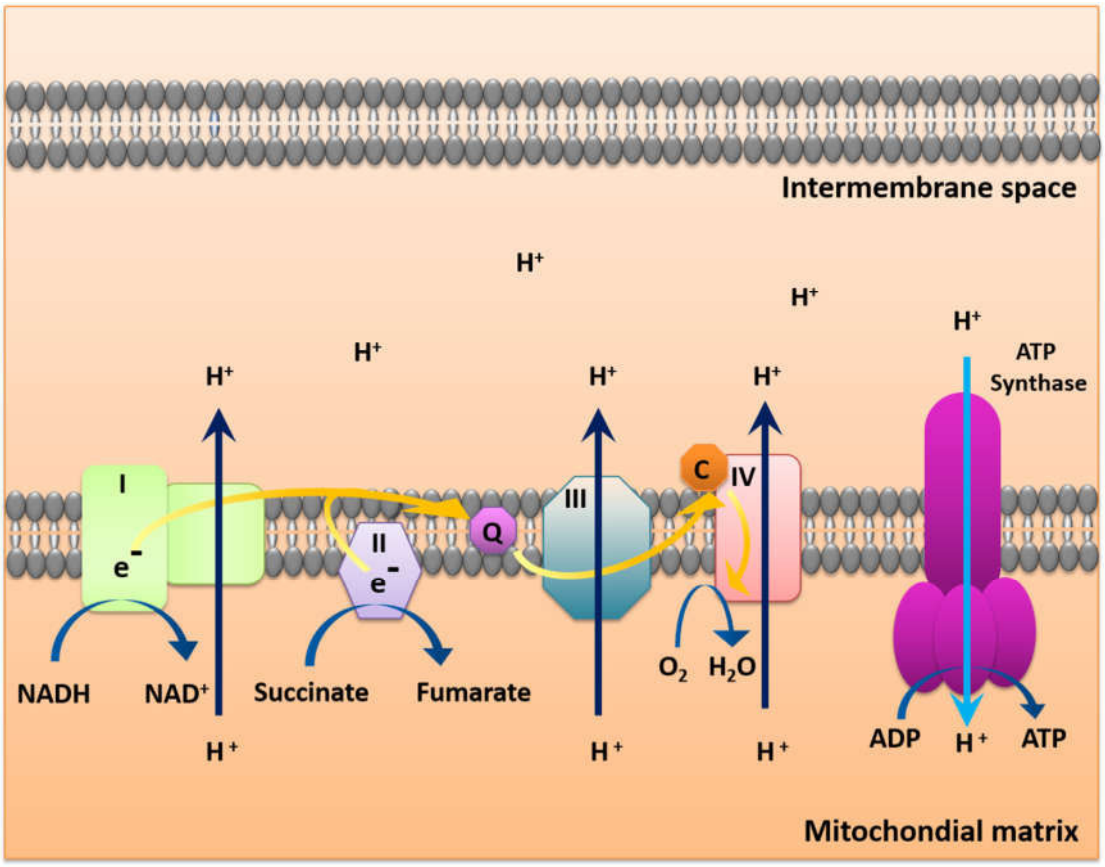


Figure 3. Electron transfer chain (ETC) of mitochondria including ATP synthase. Complex I and II transfer electrons from NADH and succinate, respectively, to coenzyme Q. The electrons move from coenzyme Q (Q) to complex III and cytochrome c (C). Complex IV accepts electrons from cytochrome c and reduces oxygen molecules to water. The resulting proton gradient is utilized to drive ATP synthase to produce ATP from ADP via oxidative phosphorylation. Modified from Schrauwen et al. [34].

These involve catalases, superoxide dismutase (SOD) and glutathione peroxidases (GPx) and non-enzymatic antioxidative mechanisms which scavenge ROS, such as ascorbic acid (vitamins C), tocopherol (vitamin E), uric acid and the endogenous molecules glutathione (GSH) [35, 36]. However, if the defensive antioxidative mechanisms are inefficient or ROS are formed in excess, the DNA, proteins and lipids can be damaged by the development of oxidative stress in biological system [37] (Figure 4).

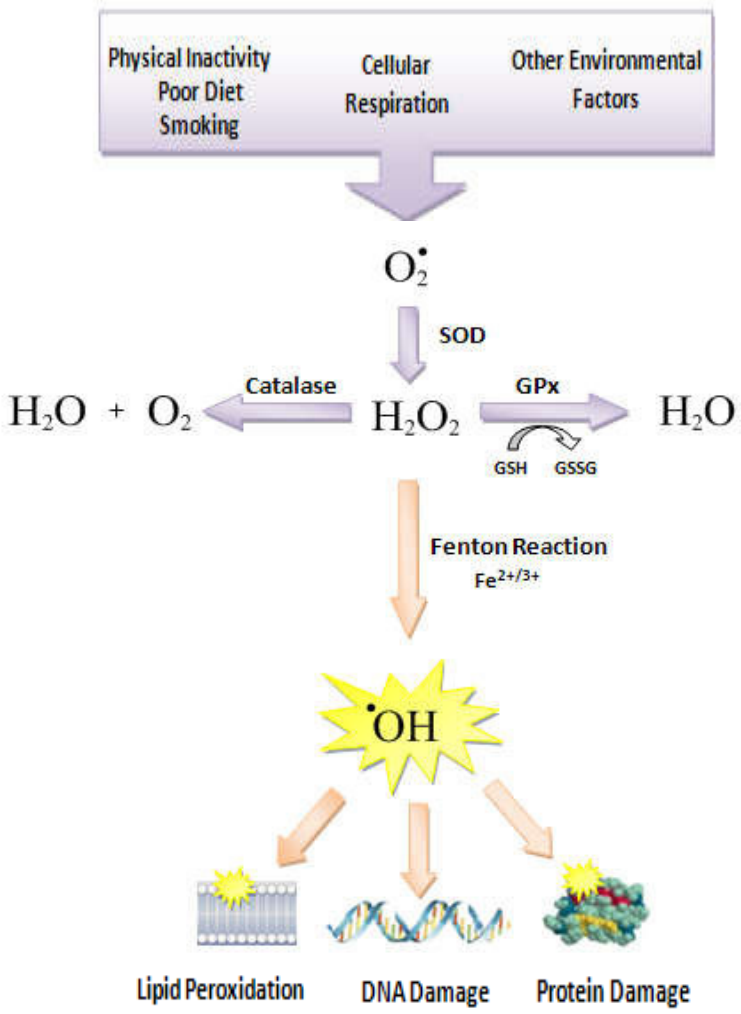


Figure 4. Generation of reactive oxygen species (ROS) and defense mechanisms against damage, exerted by superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. Modified from Roberts and Sindhu [37]

1.3 Quantification of genomic damage

Oxidative DNA damage can manifest as genomic damage [38]. Of the techniques for quantification of genomic damage, the comet assay and the micronucleus test are widely used because of the low cost and the accessibility to most academic research laboratories [39].

1.3.1 **Comet assay**

The single cell gel electrophoresis assay (SCGE) was first described by Ostling and Johanson in 1984 as a simple way of detecting DNA damage in individual cells [40]. The technique has been developed and further modified over the last three decades and is now commonly known as the comet assay [41-44]. The comet assay has become the most popular method for detecting damage of DNA and it can be used to detect damage induced by ROS for basic research on both DNA damage and repair.

In the comet assay, cells are embedded in low melting point agarose on a microscope slide, and lysed by high salt solution to remove the membrane and histones [43, 45, 46]. DNA is arranged in a tightly supercoiled form, then the supercoil relaxes, unwinding and spilling out surrounding the nucleoid, followed by electrophoresis at alkaline condition (pH>12.6) [47, 48]. The basic condition is important to accelerate the rate of unwinding and generation of broken DNA fragments, including DNA single and double-strand breaks and those developed from alkali-labile sites as well as apurinic or apyrimidinic sites.

Thereafter, the negatively charged DNA supercoils are relaxed during a brief electrophoresis and broken end fragments attracted toward the positive pole of electrophoresis field forming comet tail. On the other hand, the high-molecular-weight part, lacking free ends in the undamaged DNA, is prevented from migration within the nucleoid which appears as a comet head [47, 49] (Figure 5).

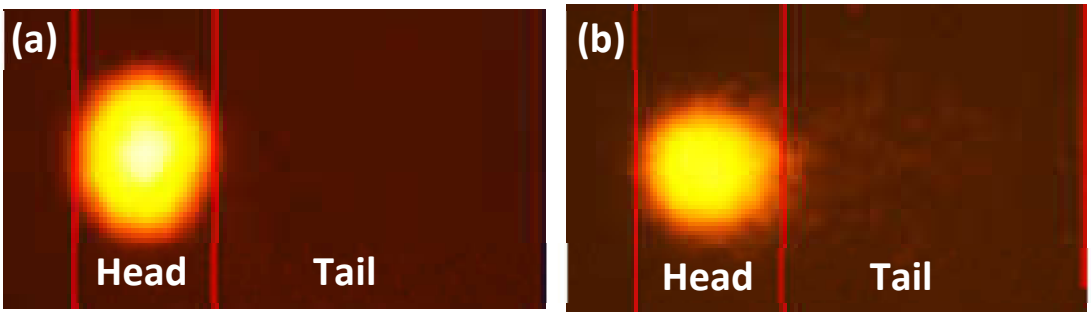


Figure 5. Representative comet assay images for a cell with intact nuclear DNA (untreated) (a) and a cell with DNA damage after insulin treatment (b). The tail represents the DNA fragments which migrate from the cell nucleus (Head).

The migration of DNA strands from individual cells can be visualized by fluorescence microscopy with a DNA staining dye such as GelRed [50], ethidium bromide [45, 46], propidium iodide [51], 4,6-diamidino-2-phenylindole (DAPI) [52] and YOYO-1 [47] or silver stain [49]. Determination of the relative amount of DNA damage can be performed in a simple way and in real time from digitized images using software developed for this purpose.

ROS play important roles in cell signaling and immune reactions as well as increase the risk of cancer due to exposure to environmental genotoxic agents e.g, chemicals and oxidizing/alkylating agents and ionizing [53]. Therefore, the comet assay is a useful investigative tool for detection of oxidation damage [54]. Moreover, the oxidative stress is associated with many human diseases e.g. diabetes, cancer, arthritis, cardiovascular and neurodegenerative diseases (reviewed in [55]).

1.3.2 **Micronucleus frequency test**

The micronucleus (MN) frequency assay is used worldwide in molecular epidemiology and cytogenetics to measure the degree of the chromosomal damage in different cell types, including peripheral blood mononuclear cells (PBMC's) after exposure to genotoxic agents or to investigate the presence of a susceptible genetic profile in human populations [56, 57].

The micronucleus (MN) originates from an acentric chromosome, chromatid fragments or whole chromosome loss events that fail to be incorporated into the main daughter nuclei during mitosis because they did not attach properly to the spindle at the anaphase stage [58-60].

These chromatin structures are eventually surrounded by a nuclear membrane and are similar in morphology to nuclei but smaller in size [61]. The formation of MN from either chromosome fragments or a whole chromosome is illustrated in Figure 6.

The cytokinesis-block micronucleus (CBMN) technique was developed to distinguish between dividing and non-dividing cells after treatment for specific scoring of MN. In

the CBMN assay, the double nucleated cells with completed nuclear division can be recognized after blocking the cell divisions by cytochalasin B [58, 62, 63].

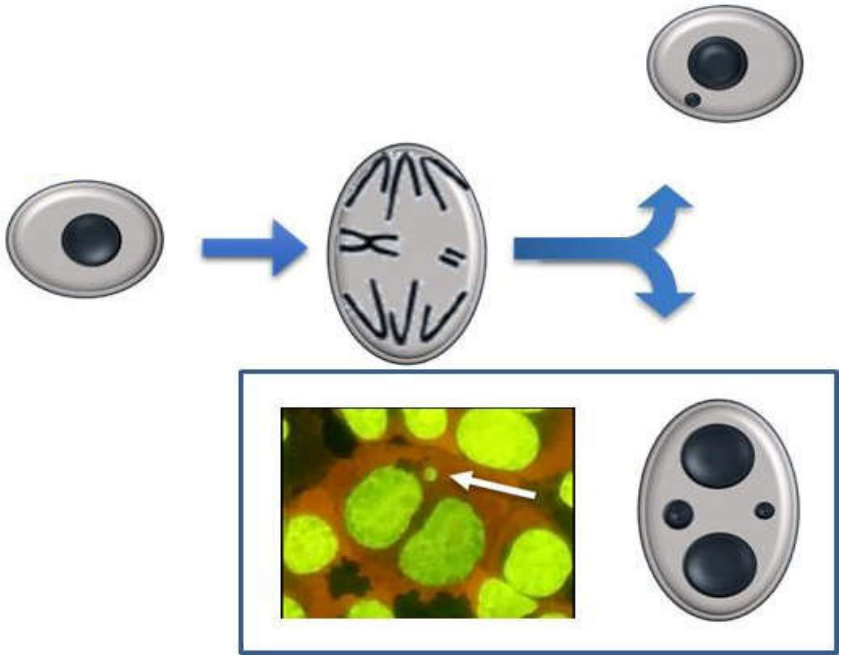


Figure 6. Micronuclei formation from chromosome fragments or lagging whole chromosomes during cell division and representative cells containing micronuclei. Modified from Fenech et al. [57]. The representative micronucleus picture is taken from our publication with permission of the journal [64].

Thus, the micronucleus frequency provides a convenient and reliable determination of both chromosome breakage and loss and has been extensively applied as a biomarker for genotoxicity assessment to measure the rates of genomic instability. Genomic instability appears to be an early step in the progress of carcinogenesis [56, 65]. Increased micronucleus frequency via chromosome instability phenotype is an expression of DNA damage such as DNA double-strand breaks and is associated with aging and high risk of cancer development [66-69].

1.4 Endocrine hormones and genotoxicity

The endocrine system is a network of glands that produce hormones such as growth hormones, 'fight-and-flight' hormones (epinephrine and norepinephrine), thyroid hormones, and steroid hormones (progesterone and estradiol) to coordinate various human functions e.g. energy metabolism, growth and development (Figure 7). These hormones play an essential role in human health and development of diseases can be related to hormone disorders and genomic damage [70, 71].

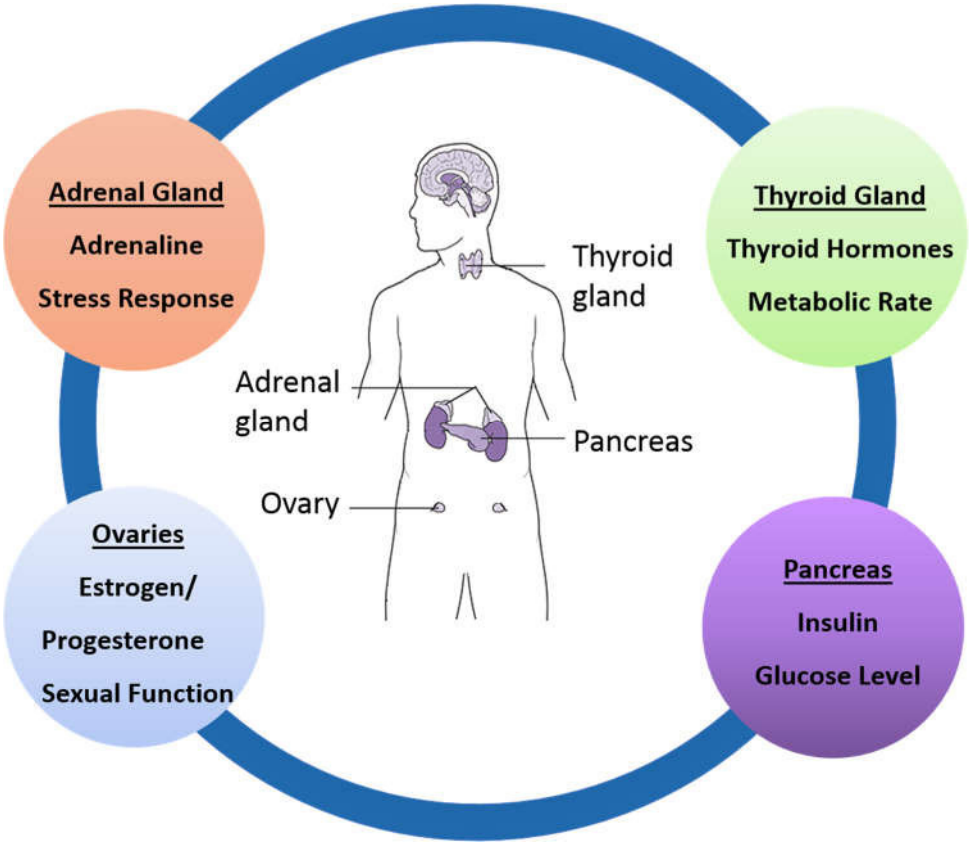


Figure 7. Glands and endocrine hormone network including growth hormones (insulin), stress hormones (adrenaline), thyroid hormones, and female steroid hormones (progesterone and estradiol) and their sources and functions. Modified from Ciccone, C. D. [72].

1.4.1 Insulin

The human insulin molecule consists of two polypeptide chains. The A chain (C-terminal) has 21 amino acid residues and B chain (N-terminal) has 30 amino acid

residues. Chains A and B contain alpha helices but no beta strands and are linked by 3 conserved disulfide bridges. The A-chain contains an intra-chain disulfide bridge between residue 6 and 11and the other two disulfide bridges keep the two chains together (Figure 8). The proinsulin has additionally the C-chain, between the chains A and B, and after proinsulin breakdown, insulin is liberated into the circulation [73]. The biosynthesis of insulin is initiated by producing preproinsulin from islet beta cells in the pancreas. Preproinsulin is rapidly converted to proinsulin by proteolytic enzymes. The later intermediate, proinsulin with a C chain connecting A and B chains, is then transformed to insulin by eliminating of C chain (Figure 9) [74].

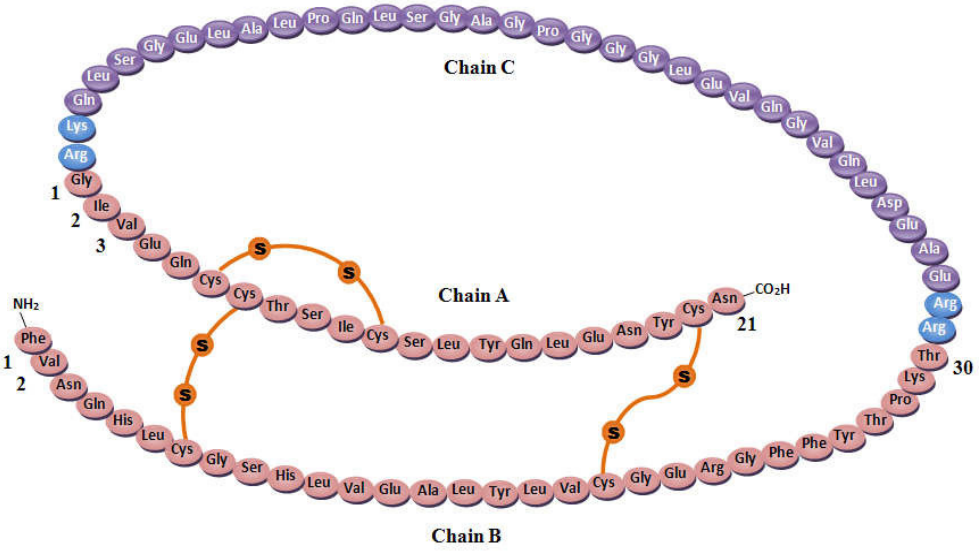


Figure 8. The structure of the human proinsulin molecule including chains A and B linked together by C peptide chain. Insulin is composed of chains A and B joined by two disulfide bonds and an additional disulfide is formed within the A chain (the amino acid residues in the A and B chains are indicated in pink). The C-peptide is represented in purple. The blue circles indicate the cleavage site for conversion from proinsulin to insulin. Modified from Joshi et al. [74].

The inert stable form of insulin is produced and stored as hexamer (composed of three insulin dimmers) binding to a Zn atom. However, the active form of insulin is slowly released as monomer from the hexamer [73].

The human insulin differs from porcine insulin in only one amino acid side chain and bovine insulin in three amino acid residues. Yet, biosynthetic human insulin is available

and produced in high purity by DNA technology due to the development of genetic engineering [75, 76].

Insulin is secreted to blood to regulate the carbohydrate and fat metabolism by absorbing glucose from the blood stream. Glucose is stored in skeletal muscles and fat tissues rather than using glycogen or fats to produce glucose under fasting conditions as a source of energy [77]. Thus, insulin is essential for maintenance of whole-body glucose homeostasis as well as controlling the signals to other body systems, such as amino acid uptake.

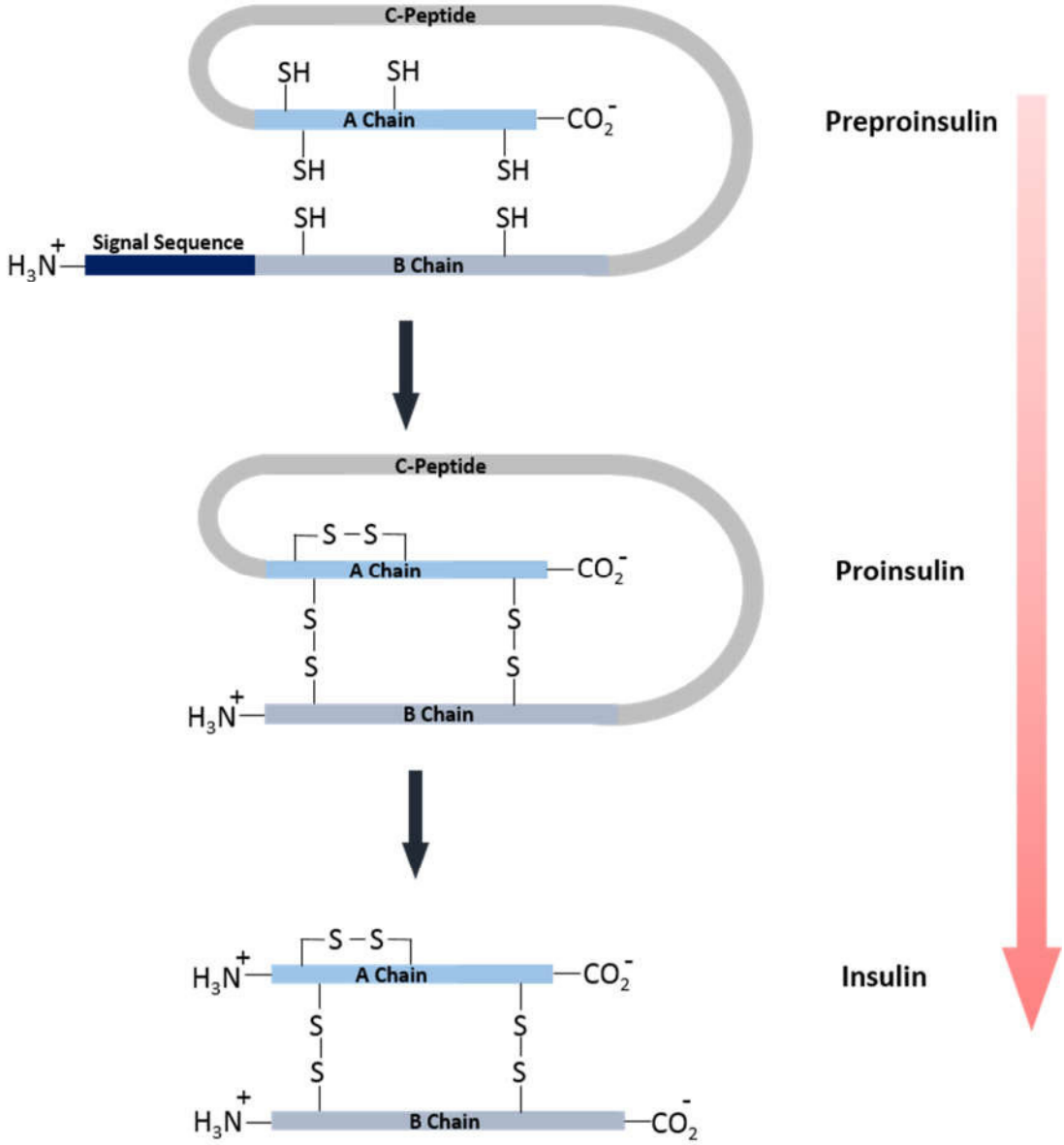


Figure 9. Biosynthesis of insulin through the inactive preproinsulin and proinsulin intermediates via initial elimination of the N-terminal signal sequence and formation of the disulfide bridges followed by releasing the intervening C-peptide. Modified from Joshi et al. [74].

1.4.1.1 **Insulin signaling pathway**

Insulin signaling is mediated by numerous steps within a highly integrated network that regulates several functions via two main signaling downstream pathways; (i) the PI3K/AKT/mTOR pathway which has a major role in the regulation of the metabolic

actions of insulin e.g. glycogen, lipid and protein synthesis, anti-lipolysis and the control of hepatic gluconeogenesis as well as translocation of GLUT4 to cell membranes [78]. (ii) The Ras–mitogen-activated protein kinase (MAPK) pathway represents the mitogenic pathway, which controls the expression of several genes, and stimulates the growth-promoting actions of insulin [79] (Figure 10).

The signaling pathway of insulin is initiated by activation of insulin receptor (IR) molecules in the cell membrane [80-82]. This binding induces a conformational change and phosphorylation of tyrosine residues which are present in the intracellular component of the insulin receptor substrate proteins (IRS 1/2). This activates phosphatidylinositol 3-kinase (PI3K) class IA which is the most important lipid kinase, among the PI3K subtypes

PI3K dysfunction is associated with many pathological conditions related to different aspects of diabetes, human cancer, and aging [83]. Therefore, targeting PI3K is one of the attractive strategies for clinical applications. Additionally, PI3K class IA consists of three different catalytic subunits (p110α, p110β and p110δ) [84, 85]. These catalytic subunits form heterodimers with one of the regulatory subunits (p85α, p85 β, p85 δ, p50 α and p55 α) [86]. The Src homology 2 (SH2) domains on the regulatory subunit facilitate the binding with phosphorylated tyrosine in receptor tyrosine kinases or IRS-1 [87].

The activated PI3K promotes the phosphorylation of phosphatidylinositol-3,4,5-biphosphate (PIP2) to phosphatidylinositol,-3,4,5-triphosphate (PIP3) at the plasma membrane followed by stimulation and translocation of the serine/threonine protein kinase AKT which activates the downstream target mTOR through direct phosphorylation and inhibition of the tuberous sclerosis complex TSC-2 [88, 89] and Ras homolog enriched in brain (Rheb) [90]. The mammalian target of rapamycin (mTOR) is a highly evolutionarily conserved serine/threonine protein kinase that plays a significant role in cell signaling pathways by regulating the phosphorylation of the required proteins for cell-cycle progression, cell growth, protein synthesis, and proliferation [91, 92].

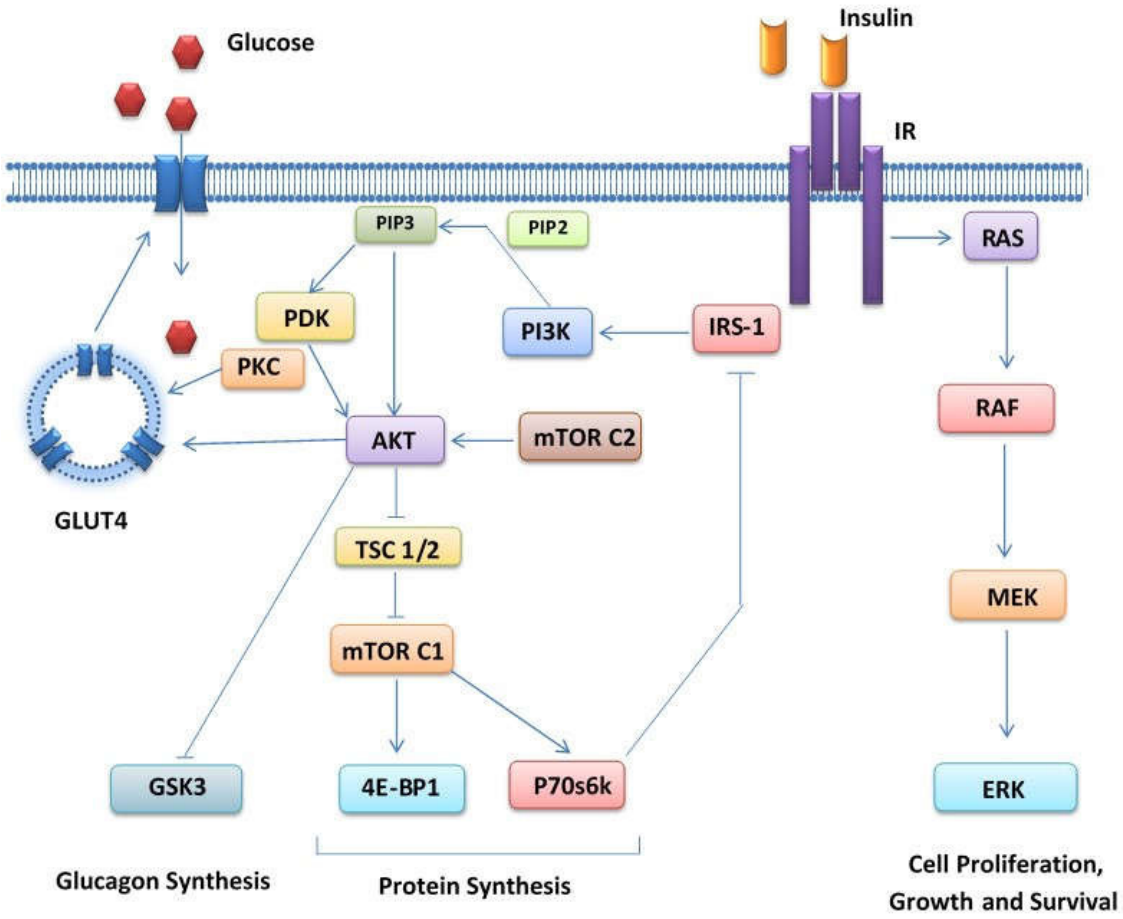


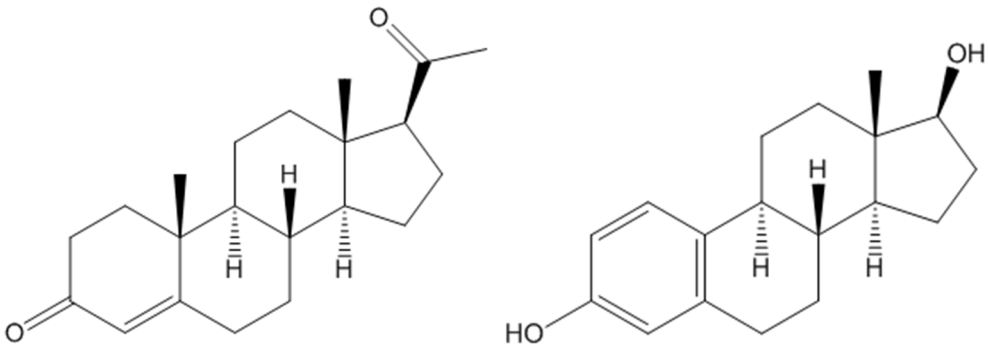
Figure 10. Insulin signaling pathway and the principal downstream components of the PI3K/AKT (metabolic) and Ras/MAP kinase (Mitogenic) pathways. Abbreviations: IR: insulin receptor; IRS-1: insulin receptor substrate 1, PI3K: phosphoinositide-3-kinase, PIP2: phosphatidylinositol-4,5-biophosohate, PIP3: phosphatidylinositol-3,4,5-triphosohate, AKT: protein kinase B, mTORC1: mammalian target of rapamycin complex 1, mTORC2: mammalian target of rapamycin complex 2, p70s6k: ribosomal protein S6 kinase1, 4E-BP1: eukaryotic initiation factor 4E (eIF-4E)-binding protein 1, PDK: 3-phosphoinositide-dependent protein kinase, PKC: protein kinase C, GSK3: glycogen synthase kinase 3, TSC1/2: tuberous sclerosis complex ½, MEK: mitogen-activated protein kinase kinase, ERK: extracellular signal-regulated kinase. Modified from Poloz and Stambolic [93]

mTOR exists as a catalytic subunit in two distinct complexes, mTORC1 and mTORC2 in which they are characterized by two different proteins known as raptor (regulatory associated subunit), and rictor (rapamycin-insensitive companion), respectively [94, 95].

mTORC1 integrates growth factors and nutrients to enhance the phosphorylation of the well-known ribosomal protein S6 kinase1 (S6K1) and eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1) to regulate transcription of mRNAs for cell cycle progression and growth [96]. However, mTORC2 had been found to affect the phosphorylation of AKT on S473 [97, 98]. The overactivation of the PI3K/AKT/mTORC1 pathway is thought to contribute to the development of human cancers [99].

1.4.2 **Progesterone and estradiol**

The steroid hormones estradiol (17β-Estradiol, E2) and progesterone (P4) (Figure 11) are mostly synthesized from cholesterol and produced primarily in the ovaries. They contain four rings of carbon atoms in their main chemical structure, while the variation of the functional groups attached to the basic steroid rings gives different functions of action.



Progesterone **Estradiol**

Figure 11. Chemical structures of the steroid hormones progesterone and estradiol.

The physiological concentrations of the steroid sex hormone E2, which is the more biologically active hormone compared with its two metabolites estrone (E1) and estriol (E3) [100], and P4 are important to promote proliferation, growth maintenance of female reproductive organs and other reproductive tissues such as the breasts, uterus, and vagina during puberty, adulthood, and pregnancy, and it is also responsible for the regulation of the menstrual cycle [101, 102]. The genomic effects of P4 and E2 are

mediated after binding to their receptors, namely the progesterone receptor (PR) and the estrogen receptor (ER), respectively [103]. The receptors are located intracellularly, and function as signal transducers and ligand-inducible transcription factors to modulate expression of target genes [104, 105].

At the center of the majority of estrogen and progesterone action are two estrogen receptor (ER) proteins (ERα and ERβ) originating from separate genes and two progesterone receptors (PRs), PRα and PRβ, which arise from differential promoter usage [106-108]. The level of female sex hormones in the body may increase due to the intake of oral contraceptives, hormone replacement therapy, and pregnancy. This elevation of hormone level may represent a risk factor in cancer incidence [101].

1.4.3 **Triiodothyronine and Thyroxine**

Triiodothyronine (T3) and thyroxine (T4) are thyroid hormones (THs) produced mainly by the thyroid gland (Figure 12). THs are important in inducing the basal metabolic rate, regulating mitochondrial functions and consumption of oxygen [109-111]. Under normal conditions, T4 plasma concentrations are in greater amount than T3 [112-114], but T3 is the active form of THs. Therefore, T3 has about 20-30 times greater affinity to bind to the thyroid hormone receptors (TRs) than T4 [115-119]. TRs are classified into two main receptors TRA and TRB [120]. Additionally, TRB can promote genes which play a significant role in mitochondrial respiration [121]. Thus, induction of respiration increases the oxidative stress produced by mitochondria that could be involved in both DNA damage induction and enhancement of aging. Moreover, there is a correlation between TRs and degenerative diseases and cancer [121].

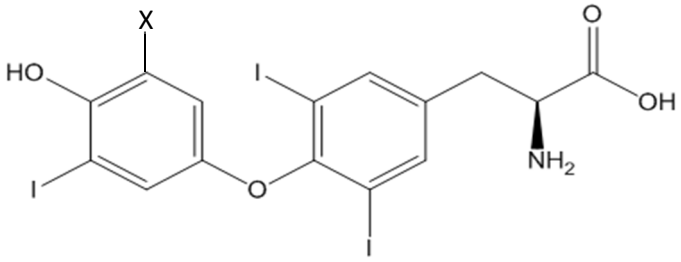


Figure 12. Chemical structures of triiodothyronine (T3, X= H) and thyroxine (T4, X= I).

1.4.4 **Adrenaline**

Adrenaline (epinephrine) is a hormone secreted by the adrenal gland especially by adrenal medulla under stress conditions (Figure 13). The main function of adrenaline is to control and regulate stress in the body even under emergency or chronic stress. Furthermore, adrenaline has several physiological functions such as regulation of cardiac stimulation, blood pressure, and regulation of the blood glucose levels to prepare the body for extraordinary physical and mental effort [122-124].

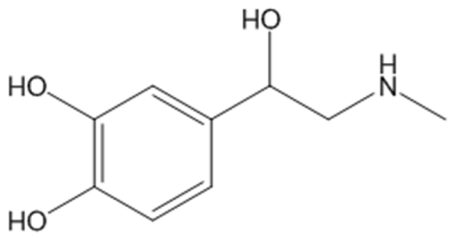


Figure 13. Chemical structure of adrenaline.

This hormone is used in the treatment of cardiac arrest, asthma, glaucoma and allergic reactions [125-127]. Since adrenaline is released to influence the physiological response to stress, the secretion of adrenaline is not constant, and the basal level of adrenaline is in the nanomolar range [128]. Under stress conditions, the body is stimulated to release excess adrenaline that can bind to adrenergic receptors which enables a quick response known as “fight and flight” response [129]. After that, the metabolic rate and fat metabolism are also increased to produce more energy [130]. Moreover, reactive oxygen species induced by excessive release of the stress hormone adrenaline may cause disruption of DNA integrity which gives rise to cancer [131].

1.5 Insulin-related diseases

Development of metabolic diseases such as type 2 diabetes, pre-diabetes and insulin resistance with obesity are associated with an abnormal response to insulin [132-135]. Therefore, insulin deficiency or excess of insulin level can affect the insulin signaling pathways and its functions [136, 137].

1.5.1 Diabetes mellitus (DM)

Diabetes mellitus (DM), is a chronic metabolic disease occurring as defects in insulin secretion and actions, known as type I (T1DM) and type II (T2DM), respectively, as defined by the World Health Organization (WHO) [138]. In the 21st century, DM is considered as one of the main global health problems, where the risk of diabetes increases due to lifestyle changes such as physical activity, stress and diet [139, 140]. Moreover, it is expected that the number of patients with DM will increase up to 366 million by 2030 [141]. Additionally, the type II diabetes patients have many severe complications, e.g. heart diseases and kidney failure, and retinopathy with increase of the risk of morbidity and mortality [142-144]. Furthermore, insulin resistance and increased oxidative stress have been observed in patients with T2DM [145-148]. Overproduction of ROS and altered cellular redox status are associated with several diseases such as hyperinsulinemia [149]. Studies suggest that hyperinsulinemia may be an essential risk factor for different cancer types [150-157]

1.5.2 Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus (GDM) is defined as clinical disorder developed during pregnancy associated with elevation of glucose level in the blood resulting in hyperglycemia. The percentage of GDM women is about 2-14% worldwide and it is increasing globally [158, 159]. Pregnancy is divided into three trimesters, and GDM is usually diagnosed at the end of the second trimester. It is accompanied by increased risk of maternal and fetal complications [160, 161]. The development of GDM can be related to several factors including (but not limited to) family history of diabetes mellitus, overweight/obesity and maternal age [162, 163]. Women with GDM history

are advised to control the blood glucose level even after delivery because they are more likely to develop cardiovascular diseases and T2DM later on [164]. Commonly, GDM is associated with high levels of oxidative stress, due to hyperglycemia that favors overproduction of reactive oxygen species [165, 166].

1.5.3 **Obesity**

Obesity is a chronic and severe disease, according to the World Health Organization (WHO), characterized by the accumulation of excess adipose tissue to more than the amount needed for the normal body function [167, 168]. It causes adverse effects on human health and increases health problems leading to morbidity and mortality [169]. Moreover, obesity is a major risk factor associated with several chronic diseases; including diabetes mellitus type II, hypertension, cardiovascular diseases and cancer [169-172]. The correlation between obesity and insulin resistance, which is the main precursor for the progress of T2DM, has been noticed and well described in those obese subjects without family history of diabetes or obesity [173-176].

The most common and simple method to measure the obesity level is the Body Mass Index (BMI) assessment, which classifies patients into groups and compares them using a mathematical formula by dividing the body weight in Kg to the second power of the height in meter [177]. The BMI is characterized as normal between 20-25 kg/m^2, while a BMI ≥ 30 kg/m^2 is considered as obese (Table 1) [168].

Table 1: Classification groups of BMI according to WHO [168].

Classification groups	**BMI (kg/m^2)**
Underweight	<18.50
Normal range	18.50–24.99
Overweight	25.00–29.99
Obese	≥30.00

2 Objectives

Diabetes mellitus and obesity are global metabolic diseases that become more frequent each year. They are also associated with hypertension, cardiovascular disease, hyperinsulinemia as well as premature mortality and morbidity. In diabetic and obese patients, hyperinsulinemia can be accompanied by an excessive increase in oxidative stress that activates signaling cascades resulted in DNA damage and mutations. Several studies proposed that hyperinsulinemia may be an important risk factor for various types of cancer.

For further investigations of the role of insulin signaling pathways in the generation of oxidative stress and genomic damage, we selected interesting natural and pharmacological compounds which can interfere with the insulin cascade including PI3K inhibitors, resveratrol, lovastatin, and the mTORC1 inhibitor RAD-001. These compounds have various beneficial effects against metabolic disorders, but their anti-genotoxic potential regarding insulin signaling had not been investigated. Therefore, this study aimed to evaluate the effect of these natural and pharmacological compounds on genomic damage and oxidative stress mediated by insulin in normal rat kidney (NRK) cells in vitro.

We furthermore investigated the possible link between elevated insulin levels and increased DNA and genomic damage in a small patient study, comparing peripheral blood cells of women affected by gestational diabetes mellitus with healthy pregnant women. Because certain steroid hormones (estradiol and progesterone) are elevated during pregnancy we decided to analyze their influence on insulin-mediated genomic damage and oxidative stress in vitro. Two non-steroidal hormones (adrenaline and triiodothyronine) were also investigated in vitro in combination with insulin because induction of oxidative stress had been described for these two hormones. For these in vitro combination treatments, we used HL-60 cells and peripheral blood mononuclear cells (PBMC's).

3 Materials and methods

3.1 Chemicals and reagents

Tempol, wortmannin, Histopaque 1077, lovastatin and resveratrol were purchased from Sigma-Aldrich (St. Louis, Missouri, or Munich, Germany). RAD-001 was purchased from MedChemTronica (Stockholm, Sweden). Human insulin, β-estradiol and progesterone were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). PI3K inhibitors were donated from Novartis (Basel, Switzerland). Triiodothyronine (T3) was purchased from Cayman Chemicals (Michigan, USA). Dihydroethidium (DHE) was purchased from Merck Biosciences GmbH (Schwalbach, Germany). GelRed and GelGreen were purchased from Biotrend (Köln, Germany). Basic laboratory chemicals were purchased from Sigma-Aldrich (Munich, Germany) and Merck Biosciences GmbH (Schwalbach, Germany). Cell culture media and reagents were obtained from PAA Laboratories GmbH (Pasching, Austria) and Invitrogen Life Technologies (Darmstadt, Germany). If not mentioned otherwise, all chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

3.2 Antibodies

Anti-AKT (pS473) antibody (22650) was purchased from Rockland (Gilbertsville, PA, USA). Anti ß-actin antibody (T6199) was purchased from Sigma-Aldrich (Taufkirchen, Germany) and the secondary antibodies HRP-conjugated goat anti-mouse IgG (sc-2005) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody HRP-conjugated anti-rabbit IgG (7074s) was purchased from Cell Signaling Technology Inc. (Beverly, USA).

3.3 Cell culture

Normal rat kidney epithelial cells (NRK) were obtained from European Collection of Authenticated Cell Culture (ECACC, Salisbury, UK) and grown in DMEM medium (4.5 g/L glucose) supplemented with 10% (v/v) fetal calf serum ,2 mM L-glutamine,

1% (w/v) non-essential amino acids (NEAA) and 1% (v/v) antibiotics. They were subcultured twice per week.

HL-60, a human promyelocytic cell line, was kindly donated by Prof Schinzel (Vasopharm GmbH) and cultured in RPMI 1640 medium supplemented with 2 mM Glutamine L-glutamine, 10 % fetal bovine serum (FBS) and 0.4% (w/v) antibiotics (50 U/mL penicillin and 50 mg/mL streptomycin). They were subcultured three times per week.

3.4 Vitality test

Cells were seeded one day before the experiment in a control medium. After treatment of the cells with the different compounds for 15 min followed by the addition of insulin for 2 h cells were harvested and 70 µL of the cell suspension was stained with 30 µL GelRed Biotrend (Köln, Germany) staining solution. Twenty microliters of this mixture were applied to the slide, and the fractions of green and red cells in a total of 200 cells were counted at a 500-fold magnification with a fluorescence microscope.

3.5 Comet assay

The alkaline version of the comet assay detects single- and double-strand breaks as well as alkali-labile lesions on an individual cell basis and is considered a standard test for genotoxicity [46]. Briefly, NRK cells were treated with different compounds as described in Table 2. After 2 h, the cells were harvested and 20 µL of the treated cells suspension was mixed with 180 µL of 0.5% low-melting agarose and added to fully frosted slides that had been covered with a bottom layer of 1.5% high melting point agarose. The slides were incubated in lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, and 10-g/L N-lauroylsarcosine sodium adjusted to pH 10 with NaOH) with 1% Triton X-100 and 10% dimethyl sulfoxide at 4 °C. After at least 1 hour, the slides were washed and then placed in the electrophoresis solution (300 mM NaOH and 1 mM EDTA [pH 13]) for 20 minutes. Then the electrophoresis was conducted for 20 minutes at 25 V (1.1 V/cm) and 300 mA. The slides were neutralized in 0.4 M Tris buffer (pH 7.5) and then dehydrated in methanol for 5 min at -20 °C. Then, the slides were dried

and stored at room temperature. After staining of each slide with 20 µL of GelRed/diazabicyclo octane (DABCO) solution for detection of DNA, images of 100 randomly selected cells (50 per replicate slide) for each sample were analyzed with a fluorescence microscope (Labophot 2; Nikon GmbH, Düsseldorf, Germany) at 200-fold magnification using image analysis software (Komet 5; BFi OPTiLAS, Gröbenzell, Germany). The percentage of DNA in the tail was used to quantify DNA migration. A representative picture of a damaged cell in the comet assay was shown in Figure 5.

Table 2: Conditions of treatment (compounds) in the comet assay.

Type of cells	* Compounds (concentrations)	Insulin concentration
NRK	Insulin (10 nM)	-
	Wortmannin (10 nM)	-
		10 nM
	PI3K inhibitor I (5, 10 nM)	-
		10 nM
	PI3K inhibitor II (5, 10 nM)	-
		10 nM
	PI3K inhibitor III (5, 10 nM)	-
		10 nM
	Resveratrol (1 µM)	-
		10 nM
	Lovastatin (10 nM)	-
		10, 100 nM
	RAD-001 (5 nM)	-
		10, 100 nM
HL-60	Insulin (10 nM)	-
	Adrenaline (0.01-100 µM)	-
		10 nM
	Triiodothyronine (0.01-100 µM)	-
		10 nM
	Estradiol (100 nM)	-
		10 nM
	Progesterone (0.01-100 µM)	-
		10 nM

(*) All compounds were added for 15 min before the addition of insulin

3.6 Micronucleus frequency test

Micronuclei are small chromatin containing structures in the cytoplasm of cells, which represent a subtype of chromosomal aberrations. This test is a standard assay in mutagenicity testing according to Organization for Economic Cooperation and Development guideline. 3×10^6 cells/mL were incubated with different substances according to Table 3. After that, the medium was removed and replaced by fresh culture medium with cytochalasin B (3 μg/mL) after washing with PBS.

Table 3: Conditions of treatments (compounds) in the micronucleus frequency test.

Type of cells	*Compounds (concentrations)	Insulin concentration	Treatment time (h)
NRK	Insulin (10 nM)	-	4
	Wortmannin (10 nM)	-	4
		10 nM	4
	PI3K inhibitor I (5, 10 nM)	-	4
		10 nM	4
	PI3K inhibitor II (5, 10 nM)	-	4
		10 nM	4
	PI3K inhibitor III (5, 10 nM)	-	4
		10 nM	4
	Resveratrol (1 μM)	-	4
		10 nM	4
	Lovastatin (10 nM)	-	4
		10, 100 nM	4
	RAD-001 (5 nM)	-	4
		10, 100 nM	4
HL-60	Insulin (10 nM)	-	24
	Adrenaline (100 nM)	-	24
		10 nM	24
	Triiodothyronine (100 nM)	-	24
		10 nM	24
	Estradiol (100 nM)	-	24
		10 nM	24
	Progesterone (100 nM)	-	24
		10 nM	24

(*) All compounds were added for 15 min before the addition of insulin

After a further 20–22 hours, cells were harvested, brought onto glass slides by cytospin centrifugation, and fixed in methanol (-20 °C) for at least two hours. Before counting, cells were stained for 3 min with GelGreen (10 µL stock solution in 990 µL distilled water), washed twice with PBS buffer, and mounted for microscopy. Due to the presence of the cytokinesis inhibitor cytochalasin B, mitosis results in double-nucleated cells. From each of 2 slides, 1000 such double-nucleated cells were evaluated for micronuclei, and the average was calculated. For substance combinations, concentrations which were described as effective in the literature and had been found not toxic in preliminary experiments were applied.

Additionally, the cytokinesis-block proliferation index (CBPI) was calculated as an assessment of potential cytostatic effects from 1000 cells per sample using the following formula: CBPI = (number of mononucleated cells + 2 × number of double nucleated cells + 3 × number of multinucleated cells) / (mononucleated + double nucleated + multinucleated cells).

3.7 Microscopic analysis of the formation of reactive oxygen species

Evaluation of the formation of ROS was performed using the cell-permeable fluorogenic probe DHE. One day before the experiment, 2×10^5 cells were seeded in 24-mm coverslips in 6-well plates in 3 mL medium; after treatment of the NRK cells with the selected compounds and 10 µM DHE, the cells were incubated in the dark at 37°C for 30 min. After that, ROS production was detected after washing with 500 µL PBS. The coverslips were mounted on a slide and observed under an Eclipse 55i microscope (Nikon GmbH) and a Fluoro Pro MP 5000 camera (Intas Science Imaging Instruments GmbH, Göttingen, Germany) at 200-fold magnification. All DHE staining images were taken using the same exposure time. Quantification was carried out by measuring gray values of 200 cells per treatment using ImageJ 1.40g (http://rsb.info.nih.gov/ij/).

3.8 Ferric reduction antioxidant power (FRAP)

To exclude interference of potential antioxidant capacity of the compounds used for pharmacological inhibition with ROS-induced genotoxicity quantification, all the inhibitors and antagonists were assessed for antioxidant activity in the cell-free system using the ferric reducing antioxidant of plasma (FRAP) method [178] which determines the reduction of a ferric tripyridyltriazine complex to its colored form ferrous, in the presence of antioxidants. Thus, in this assay, the capacity of the tested compounds to reduce Fe^{3+} to Fe^{2+} was measured, which is considered to be an important parameter for antioxidant function.

Briefly, 20 µL of sample was added to 180 µL of water. Next, 600 µL of the FRAP reagent (1:1:10 mixture of 10 mM ferric tripyridyltriazine, 20 mM ferric chloride and 300 mM acetate buffer) was added and the absorption at 593 nm was measured after 3 min. The results were quantified according to a standard curve produced using different concentration of ferrous sulfate.

3.9 Western blot analysis

After treatment of cells with insulin, resveratrol (1 µM), lovastatin (10 nM) or RAD-001 (5 nM) and the combinations with insulin for 2 h, cells were harvested and lysed in Ripa buffer, which contained freshly added protease inhibitor cocktail (PIC), sodium orthovanadate and sodium fluoride to inhibit protease and phosphatase activity. The homogenization process was facilitated by mechanical disruption of the cell membranes and the obtained suspension was then centrifuged at 14000 rpm for 30 min at 4 °C. The protein containing supernatant was transferred to a clean tube and the concentration of protein in this solution was determined using Bradford's method. Generally, 30 µg of protein per sample was loaded on acrylamide gel. After electrophoresis, the gel was blotted on PVDF membrane. The membrane was blocked overnight in either 5% bovine serum albumin for p-AKT, 5% nonfat milk powder for β-actin in TBS-T buffer (5 mM TRIS, 150 mM NaCl, 0.05% Tween-20) and then incubated with primary antibody (p-AKT (1:2000) and β-actin (1:5000)). After that the excess of primary antibody was washed off for 3 × 10 min with TBS-T buffer, then the horseradish peroxidase (HRP)

conjugated secondary antibody was added followed by washing 3 × 10 min with TBS-T buffer again. After incubation with HRP substrate, the membrane was exposed to an X-ray sensitive film and the film was developed afterward.

3.10 Human subjects

In our study, 64 samples were collected related to categories as summarized in Table 4. Blood samples were collected from the examined groups to investigate the DNA and genomic damage level in relation to gestational diabetes, which is characterized by elevated blood insulin levels. The slides were prepared from whole blood and from isolated peripheral blood mononuclear cells (PBMC's) for comet assay and micronucleus frequency test and analyzed by the author of this thesis.

The study was approved by the University of Würzburg ethics committee (no. 28/15) and was carried out according to the declaration of Helsinki. A written consent was obtained from all participants prior to enrollment in the study.

Table 4: Illustration of the study groups and the number of samples (i.e. individuals) in each group.

Groups	Conditions	Number of samples
1. Non-pregnant	-	32
2. Healthy pregnant	During pregnancy	13
	After birth	6
3. Gestational diabetics mellitus	During pregnancy	7
	After birth	6

3.11 Collection of blood samples and Isolation of human peripheral blood mononuclear cells

Blood samples were collected from several groups summarized in table 4 via an indwelling cannula and collected in coded tubes containing heparin. Samples were transported from the gynecologist`s practice (Dr. med. A. Kaufhold-Moore), Wuerzburg (Germany) to the Department of Toxicology at the University of Wuerzburg (Germany) at room temperature within 0.5-1 h and processed immediately.

100 µL from the whole blood was collected and the lymphocytes isolation was carried out using Hettich centrifuges Universal K2S (Kirchlengern, Germany). 8 mL of blood was collected into Heparin-EDTA tubes. Blood was layered 1:1 over warmed up histopaque-1077 slowly and centrifuged 400 × g (1600 rpm) at room temperature for 30 min. The mononuclear cells collected from the plasma/ Histopaque interface were washed twice with 10 mL lymphocyte medium RPMI 1640 medium (supplemented with 15% (v/v) fetal bovine serum (FBS), 1% (w/v) L-glutamine, 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, 1% (w/v) antibiotics (50 U/mL penicillin, 50 mg/mL streptomycin) and 0.1% tylosin (8 mg/mL)) and centrifuged 250 × g (1300 rpm) at room temperature for 10 min, then finally cells were resuspended in 3 mL of lymphocyte medium and counted.

Furthermore, the 3×10^5 cells were used directly, while the rest of the PBMC's were stimulated in 20 mL medium with 1 mg/mL (200 µL) phytohaemagglutinin (PHA) for 42-44 hours. After that, the cells were resuspended in fresh medium and cultured to be treated with different substances for further experiments.

3.12 Comet assay for blood samples

The comet assay was carried out as mentioned in section 3.5 with some modifications. 100 µL from the whole blood were transferred to a 0.5 mL Eppendorf tubes and the rest of the whole blood was used to collect the PBMC's as described before in section 3.13. PBMC's cells were seeded in plates. If possible, two samples from whole blood and two samples of PBMC's were prepared. This enabled in addition to the assessment of the untreated samples a positive control treated with NQO (0.5 µM; 20 min). For the

comet assay, 5 µL of the whole blood were mixed with 200 µL 0.6% low-melting-point agarose (LMP) and added to fully frosted slides that had been covered with a bottom layer of 1.5% high melting point agarose. Moreover, 3×10^5 cells of unstimulated PBMC's were resuspended in 2 mL medium. 20 µL of these cells were mixed with 180 µL 0.8% LMP agarose and applied directly to the fully frosted slides covered with a bottom layer of 1.5% high melting point agarose.

As we mentioned before that the rest of PBMC's were stimulated with the PHA in 20 mL medium for 42-44 h. After this treatment time, the cells were resuspended in fresh medium. 3×10^5 of the stimulated PBMCs cells were seeded in 12-well plates and treated with 100 nM adrenaline, T3, β-estradiol, progesterone and 10 nM insulin for 2 h. Then, samples were prepared for the comet assay as described before. Additionally, after staining of each slide with 20 µL of GelRed/diazabicyclo octane (DABCO) solution for detection of DNA, images of 50 randomly selected cells (25 per replicate slide) for each sample were analyzed with a fluorescence microscope (Labophot 2; Nikon GmbH, Düsseldorf, Germany) at 200-fold magnification using image analysis software (Komet 5; BFi OPTiLAS, Gröbenzell, Germany). The percentage of DNA in the tail was used to quantify DNA migration.

3.13 Micronucleus frequency test for blood samples

After stimulation of the cells with PHA for 42-44 h, 3×10^6 cells were cultured in 12-well plates and incubated directly with 100 nM adrenaline, T3, β-estradiol, progesterone and 10 nM insulin for h in 2 mL medium before the addition of cytochalasin B (3 µg/mL) for additional 20 h. After the treatment time, cells were harvested, applied onto glass slides by cytospin centrifugation, and fixed in methanol (-20 °C) for at least two hours. Before counting, cells were stained for 3 min with GelGreen (10 µL stock solution in 990 µL distilled water), washed twice with PBS buffer, and mounted for microscopy. In addition, the cytokinesis-block proliferation index, CBPI was determined from 1000 cells of each sample. From each of 2 slides, 1000 binucleated cells were evaluated for micronuclei, and the average was calculated.

3.14 Statistics

All data are expressed as mean ± standard derivation (SD) of the three single (independent) experiments. The data were analyzed with SPSS 22 software. In the single experiments, means of % tail DNA calculated from all 100 cells per treatment were used in comet assays. Numbers of micronucleus containing cells per 1000 cells (derived from counting 2000 cells) were used in the micronucleus frequency test. The Mann-Whitney U-test was used to determine the significance between two treatments. All results were considered significant if $P \leq 0.05$.

4 Results

4.1 PI3K inhibitors

4.1.1 Vitality test for the specific PI3K inhibitors

We tested the known PI3K inhibitor wortmannin, three additional PI3K inhibitors from Novartis company and their combinations with insulin for cytotoxicity. The compounds from Novartis company were donated to us without identification of the chemical structure. There were no cytotoxic effects with the indicated concentrations or combinations (Table 5).

Table 5. The viability of NRK cells treated with wortmannin, PI3K inhibitor 1, 2 and 3, insulin and combinations with insulin.

Treatment	Viability (%)
Control	97.17 ± 0.58
10 nM Inhibitor 1	97.17 ± 1.44
10 nM Inhibitor 2	97.50 ± 0.87
10 nM Inhibitor 3	98.17 ± 0.76
10 nM Insulin	96.83 ± 0.29
Wortmannin +Insulin	98.00 ± 0.87
Inhibitor 1 + Insulin	97.83 ± 1.25
Inhibitor 2 + Insulin	98.50 ± 1.23
Inhibitor 3 + Insulin	97.00 ± 0.00

4.1.2 **The intrinsic antioxidant capacity**

The intrinsic antioxidant capacity of wortmannin and PI3K inhibitors 1, 2 and 3 were assessed in a cell-free system by ferric reducing antioxidant power (FRAP) assay using tempol (50 μM) as a positive control. The results showed that all the tested compounds exhibited no significant increase over control (data not shown).

4.1.3 **The influence of PI3K inhibitors in the comet assay**

Wortmannin and the three additional PI3K inhibitors from Novartis company were used to investigate the potential of these inhibitors to attenuate the ability of insulin to induce strand breaks. All inhibitors at concentrations of 10 nM (Figure 14) and 5 nM (Figure 15) led to the reduction of DNA damage induced by insulin. Moreover, 10 nM concentration of wortmannin was applied related to previous dose-response experiment.

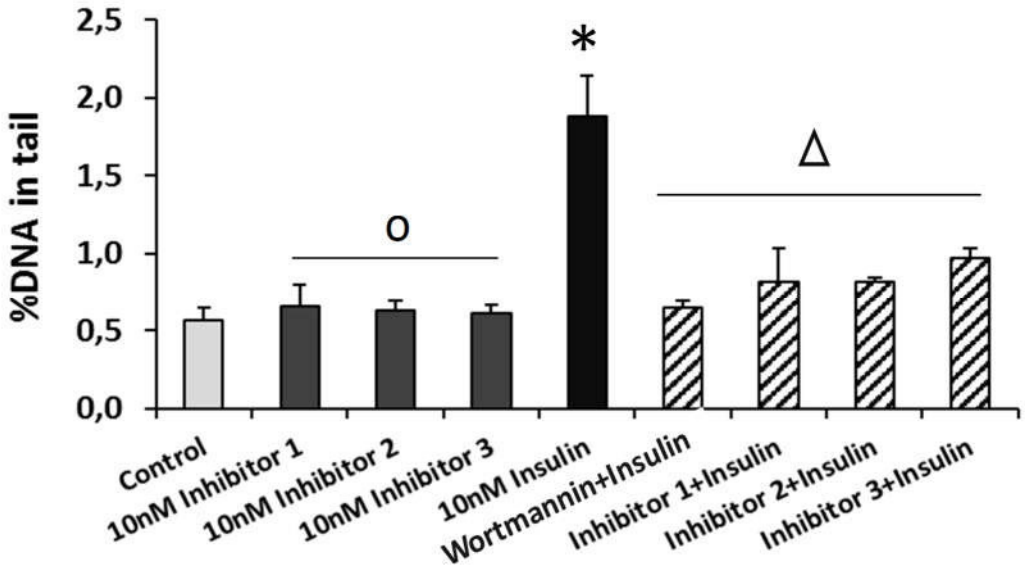


Figure 14. DNA damage (%DNA in tail) was measured by comet assay in NRK cells treated with 10 nM wortmannin,10 nM PI3K inhibitor 1, 10 nM PI3K inhibitor 2 and 10 nM PI3K inhibitor 3 for 15 min. then addition of 10 nM insulin for 2 h. 10 nM Concentration of wortmannin was applied related to previous dose response experiment. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control.

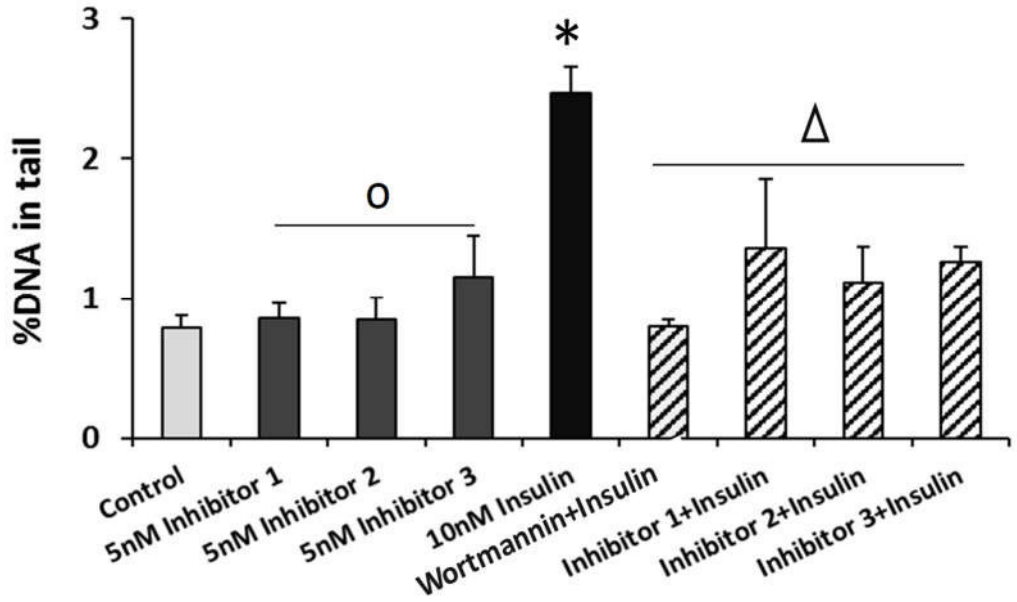


Figure 15. DNA damage (%DNA in tail) was measured by comet assay in NRK cells treated with 10 nM wortmannin, 5 nM PI3K inhibitor 1, 5 nM PI3K inhibitor 2 and 5 nM PI3K inhibitor 3 for 15 min. then addition 10 nM insulin for 2 h. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control.

4.1.4 **The influence of PI3K inhibitors on micronucleus formation**

The exposure of NRK cells with 10 nM insulin enhanced the formation of micronuclei after 4 h followed by additional 22 hours expression time. Pre- and co-incubation of NRK cells with (10 and 5 nM) of PI3K inhibitors before and during the addition of 10 nM insulin reduced micronucleus formation induced by insulin (Figure 16 and Figure 17). Additionally, the cell proliferation (CBPI) was unaffected.

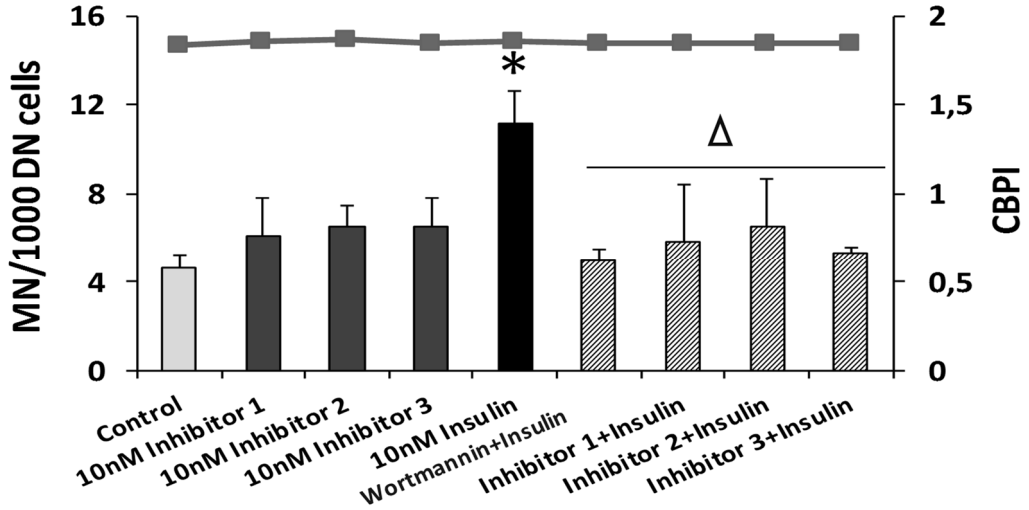


Figure 16. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in NRK cells treated with 10 nM wortmannin,10 nM PI3K inhibitor 1, 10 nM PI3K inhibitor 2 and 10 nM PI3K inhibitor 3 for 15 min. before and during the addition of 10 nM insulin for 4 hours. Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

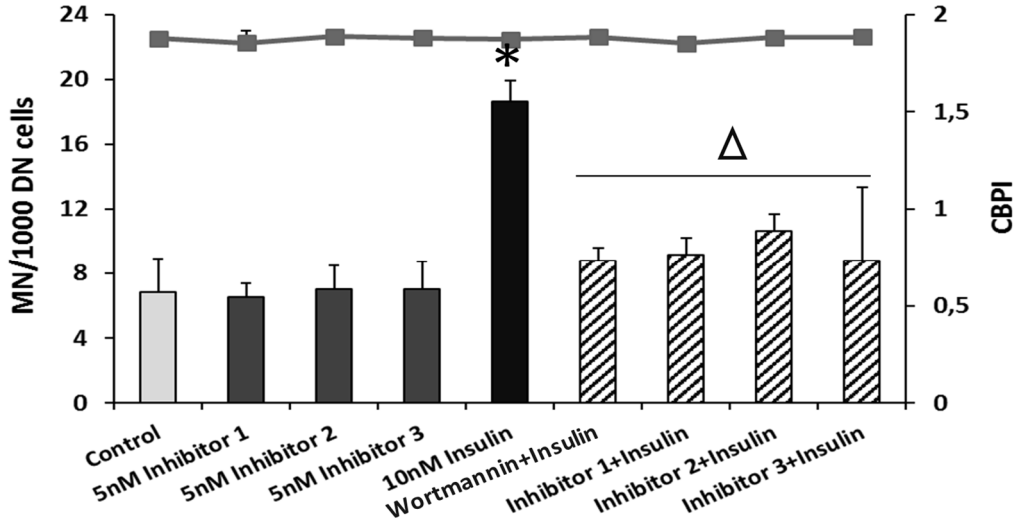


Figure 17. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in NRK cells treated with 10 nM wortmannin, 5 nM PI3K inhibitor 1, 5 nM PI3K inhibitor 2 and 5 nM PI3K inhibitor 3 for 15 min. before the addition of 10 nM insulin for 4 hours. Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.1.5 The influence of PI3K inhibitors on insulin-mediated oxidative stress

DHE measurement for ROS production showed that 10 nM of wortmannin and the three other PI3K inhibitors reduced the oxidative stress (Figure 18).

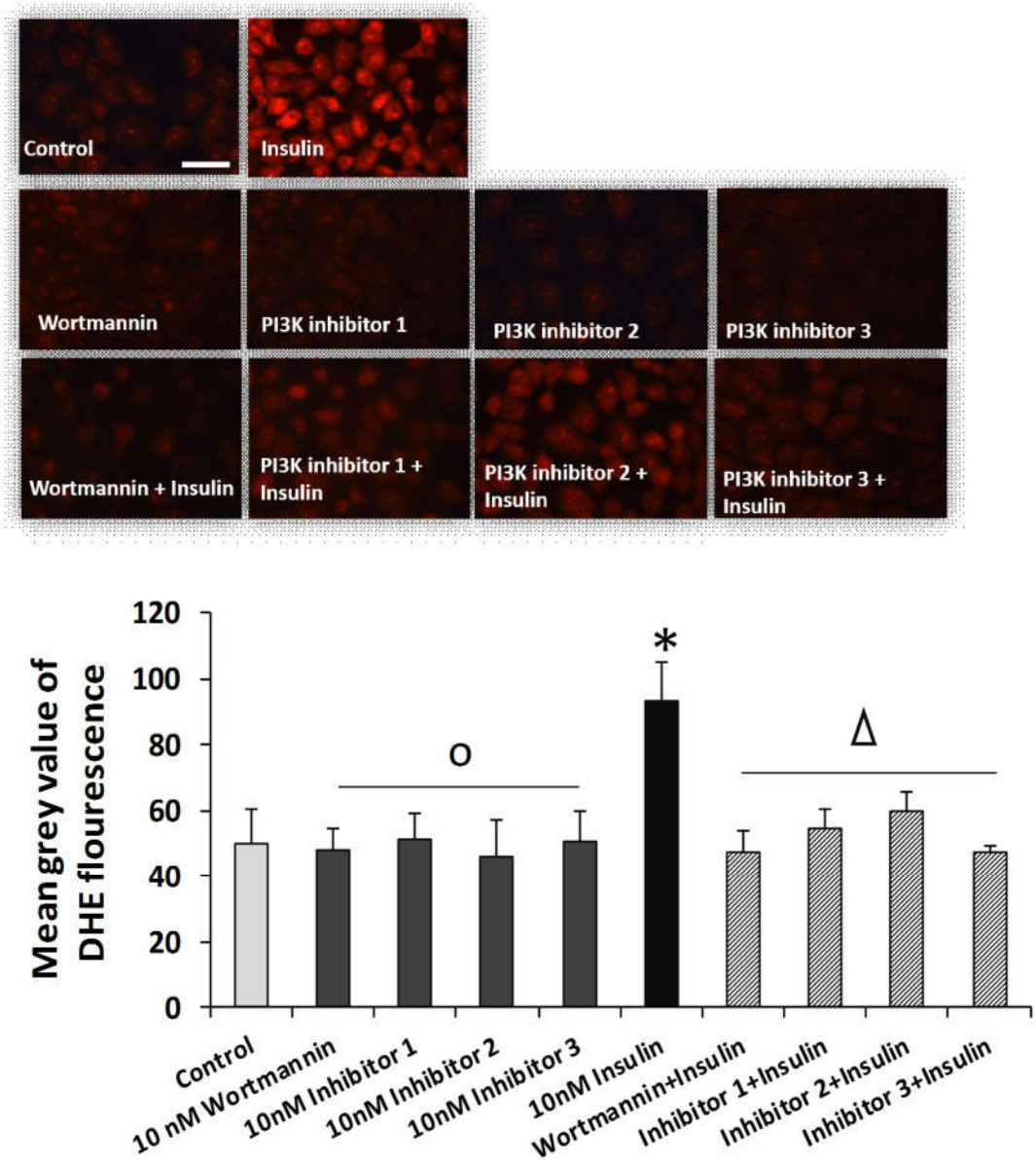


Figure 18. Microscopic detection of superoxide formation using the DHE dye in NRK cells treated with 10 nM wortmannin,10 nM PI3K inhibitor 1, 10 nM PI3K inhibitor 2 and 10 nM PI3K inhibitor 3 for 15 min. then addition of 10 nM insulin for 30 min. in the presence of DHE. Quantification of DHE fluorescence was done by measuring the mean grey value of 200 cells using image j software. (*) Significantly different from control and (Δ) significantly different from insulin. Scale bar 50 µm.

4.2 Resveratrol, lovastatin and the mTORC1 inhibitor RAD-001

Results of this paragraph have been published [64]. Some figures and text parts are taken from the publication with permission of the journal.

4.2.1 Vitality test for resveratrol, lovastatin and the mTORC1 inhibitor RAD-001

In order to study the influence of resveratrol, lovastatin, and RAD-001 on insulin-mediated cellular effects, vitality tests for cultured normal rat kidney (NRK) cells were performed. The NRK cells were treated with resveratrol (1 μM), lovastatin (10 nM) or RAD-001 (5 nM) for 15 min, then treated with insulin (10 and 100 nM) for 2 h. After that, the viable and non-viable cells percentages were quantified.

Under the experimental conditions, NRK cells did not show significant decrease in viability for resveratrol, lovastatin, RAD-001, insulin and their combinations after 2 h treatment time compared with the corresponding controls except for the RAD-001 combination with insulin. Yet, with values of still >95% viable cells, in the similar range values as all other treatments, the statistical significance in this case is not biologically meaningful (Table 6) [64].

Table 6. Viability of NRK cells after treatment with resveratrol, lovastatin, RAD-001, insulin and the combinations with insulin [64].

	Treatment	Viability (%)
Group I	Control	95.33 ± 0.58
	Resveratrol	96.12 ± 1.53
	10 nM Insulin	97.00 ± 1.00
	100 nM Insulin	95.83 ± 1.15
	Resveratrol + 10 nM Insulin	97.12 ± 1.53
	Resveratrol + 100 nM Insulin	97.67 ± 1.15
Group II	Control	95.17 ± 1.53
	Lovastatin	95.17 ± 0.58
	10 nM Insulin	97.38 ± 1.15
	100 nM Insulin	96.34 ± 1.53
	Lovastatin + 10 nM Insulin	97.12 ± 1.15
	Lovastatin + 100 nM Insulin	95.67 ± 1.53
Group III	Control	98.00 ± 1.00
	RAD-001	97.67 ± 0.83
	10 nM Insulin	98.17 ± 1.53
	RAD-001 + 10 nM Insulin	95.67± 1.53

4.2.2 **The intrinsic antioxidant capacity**

The intrinsic antioxidant capacities of the three compounds resveratrol, lovastatin and RAD-001 were determined in a cell-free system using ferric reducing antioxidant power (FRAP) assay and tempol (50 μM) as a positive control. The results showed that resveratrol at 1 μM yielded a significant increase (equivalent to 3.58 ± 0.19 μM Fe-reduction capacity) in absorption over control [64]. While lovastatin and RAD-001 exhibited no intrinsic antioxidant activity (no significant increase over control; data not shown).

4.2.3 The effect of resveratrol, lovastatin and mTORC1 inhibitor RAD-001 in the comet assay

To investigate the potential protective effects of resveratrol, lovastatin and RAD-001 against DNA damage, comet assay and micronucleus frequency test were carried out. At the beginning, 10 and 100 nM concentrations of insulin (resveratrol and lovastatin) were used. We expected to obtain additional damage with higher concentration than the physiological concentrations, but the results showed no significant difference. Thus, only 10 nM insulin in combination with RAD-100 was applied because it is much closer to physiological levels and subsequently more relevant. However, a significant induction of DNA damage was obtained after treatment of the cells with insulin for 2 h in the comet assay compared to the control cells, while the cells reacted with significant reduction of DNA damage after the addition of resveratrol, lovastatin or RAD-001 for 15 min before the treatment with insulin for 2 h (Figure 19, Figure 20 and Figure 21, respectively) [64].

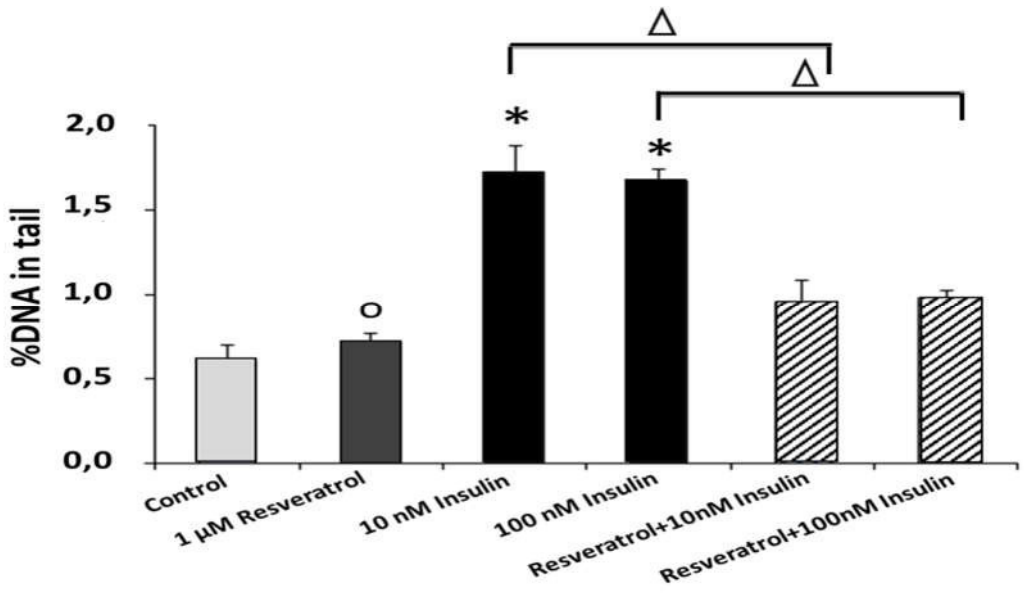


Figure 19. The DNA damage (% DNA in tail) measured by comet assay after treatment of NRK cells with resveratrol (1 nM) for 15 min before the addition of insulin (10 nM) for 2 hours. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not-significantly different from control [64].

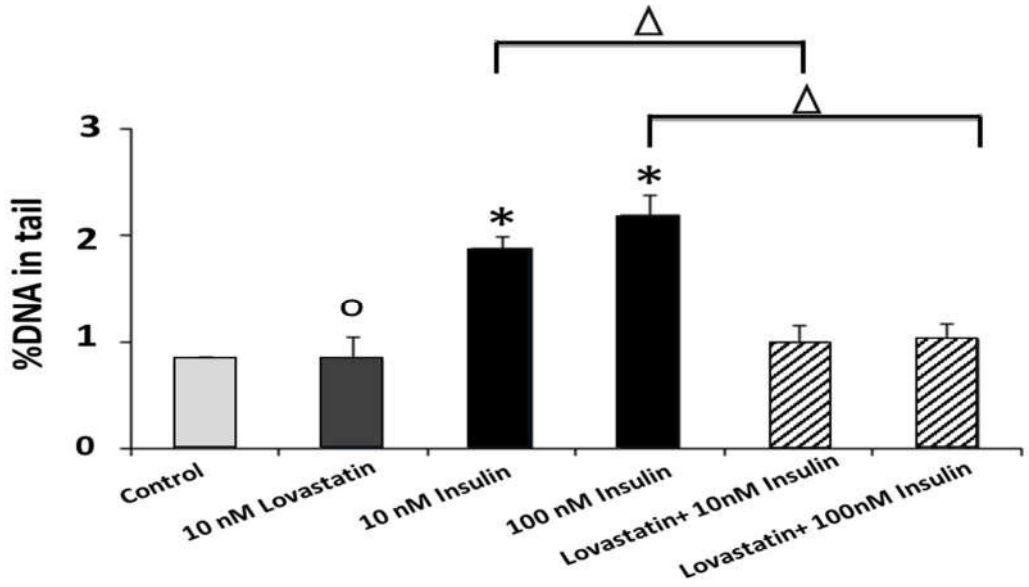


Figure 20. The DNA damage (% DNA in tail) measured by comet assay after treatment of NRK cells with lovastatin (10 nM) for 15 min before the addition of insulin (10 nM) for 2 hours. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control [64].

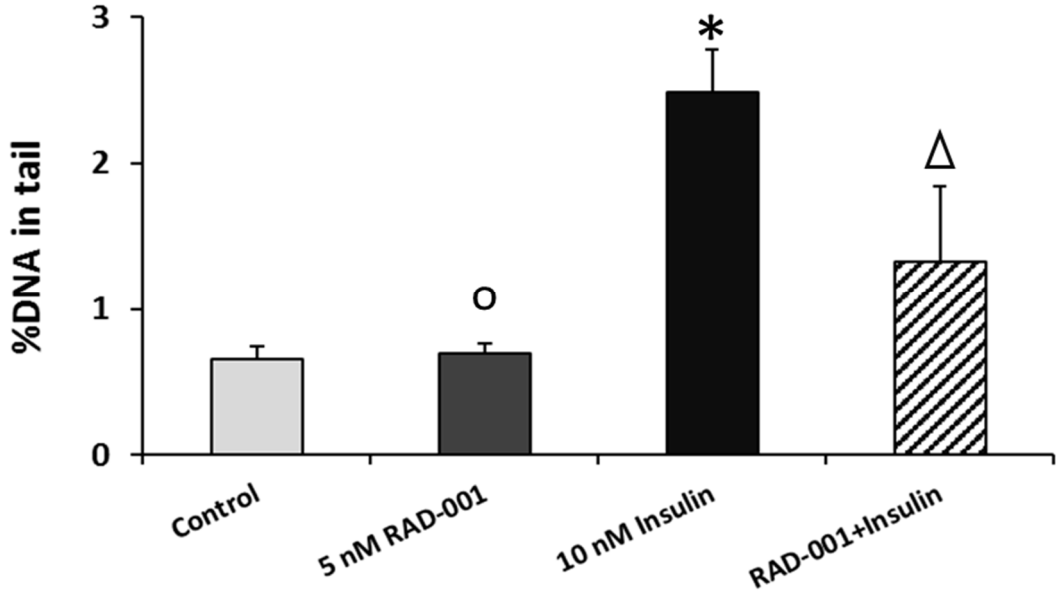


Figure 21. The DNA damage (% DNA in tail) measured by comet assay after treatment of NRK cells with RAD-001 (5 nM) for 15 min before the addition of insulin (10 nM) for 2 hours. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control [64].

4.2.4 **The effect of resveratrol, lovastatin and RAD-001 on genomic damage**

The micronucleus induction assay was performed with 10 nM and 100 nM insulin in NRK cells for 4 h treatment time. In addition, the treatment with resveratrol, lovastatin or RAD-001 compounds for 15 min before the addition of insulin suppressed the formation of micronuclei enhanced by insulin (Figure 22, Figure 23 and Figure 24, respectively). Moreover, the cell proliferation index (CBPI) was unaffected [64].

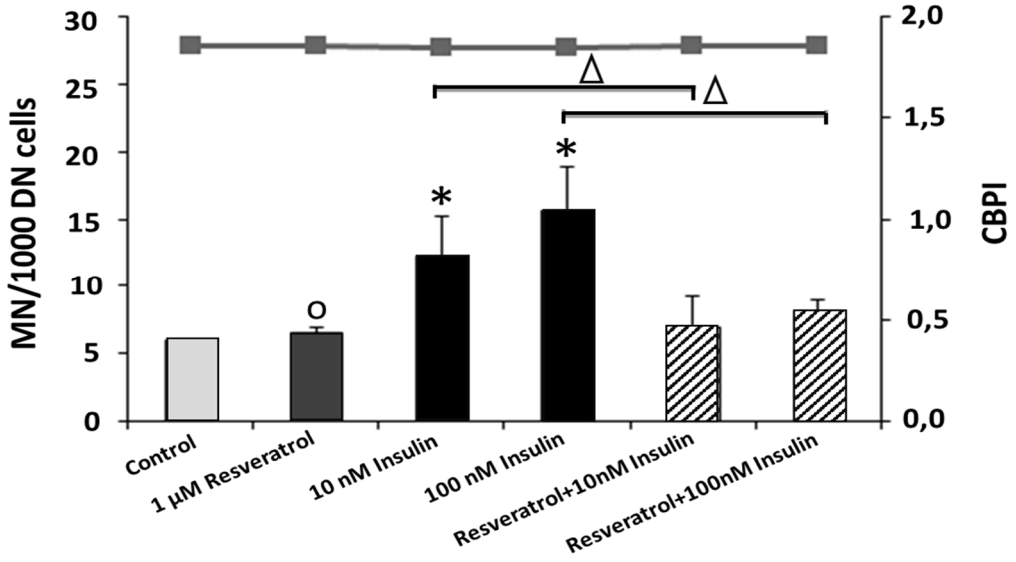


Figure 22. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in NRK cells treated with 1 μM resveratrol for 15 min. before the addition of insulin for 4 hours. Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis [64].

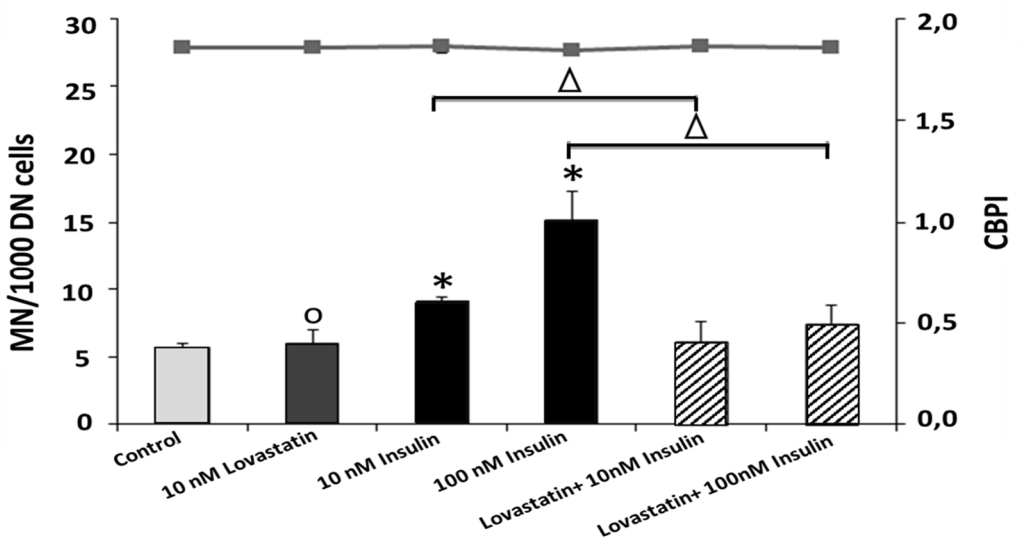


Figure 23. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in NRK cells treated with 10 nM lovastatin for 15 min. before the addition of insulin for 4 hours. Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis [64].

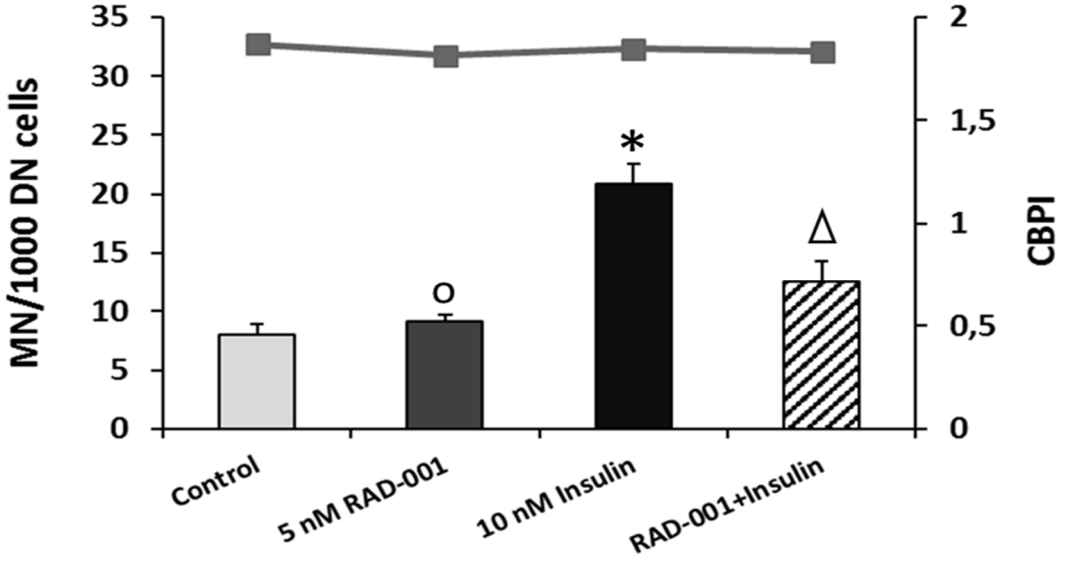


Figure 24. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in NRK cells treated with 5 nM RAD-001 for 15 min. before the addition of insulin for 4 hours. Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis [64].

4.2.5 The effect of the natural compounds resveratrol and lovastatin on oxidative stress

In order to examine the potential antioxidant activity of resveratrol, lovastatin and RAD-001 in a cellular system, the cells were treated with insulin (10 nM) for 30 min and reactive oxygen species (ROS) were detected. While the treatment of cells with resveratrol, lovastatin or RAD-001 did not increase significantly the ROS production in comparison to control. Prior addition of resveratrol, lovastatin or RAD-001 to NRK cells reduced the ROS production mediated by insulin (Figure 25, Figure 26 and Figure 27, respectively) [64].

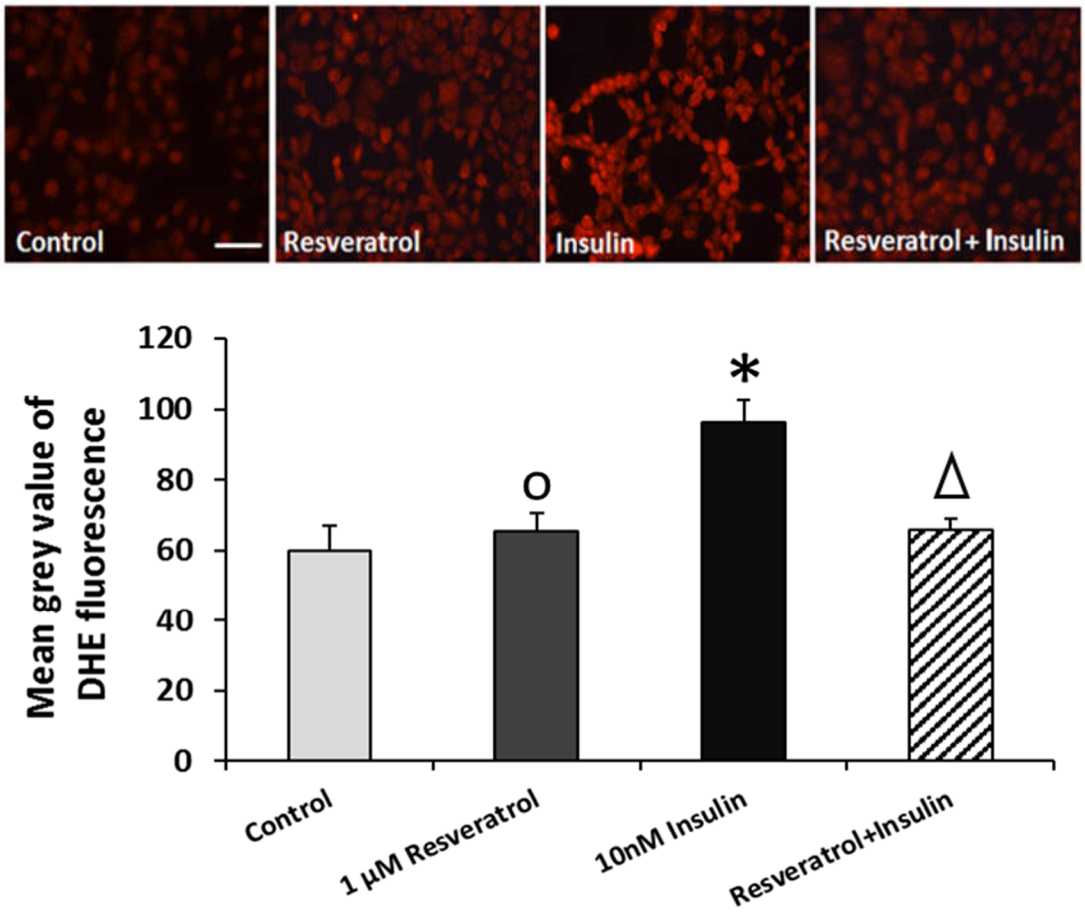


Figure 25. Microscopic detection of superoxide formation using the dye DHE in NRK cells treated for 15 min with 1 µM resveratrol then addition of 10 nM insulin for 30 min in the presence of DHE. Quantification of DHE fluorescence was done by measuring the mean grey value of 200 cells using image j software. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. Scale bar 50 µm [64].

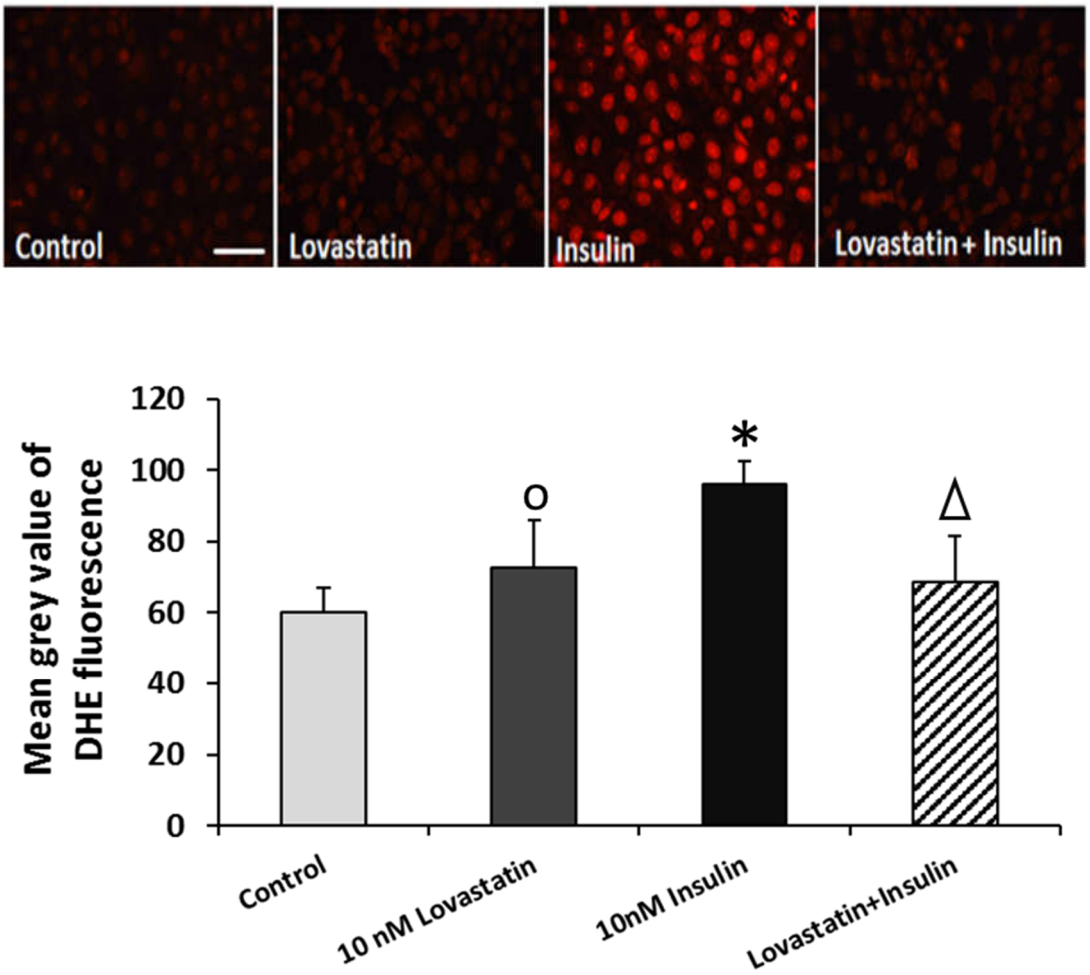


Figure 26. Microscopic detection of superoxide formation using the dye DHE in NRK cells treated for 15 min with 10 nM lovastatin then addition of 10 nM insulin for 30 min in the presence of DHE. Quantification of DHE fluorescence was done by measuring the mean grey value of 200 cells using image j software. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. Scale bar 50 μm [64].

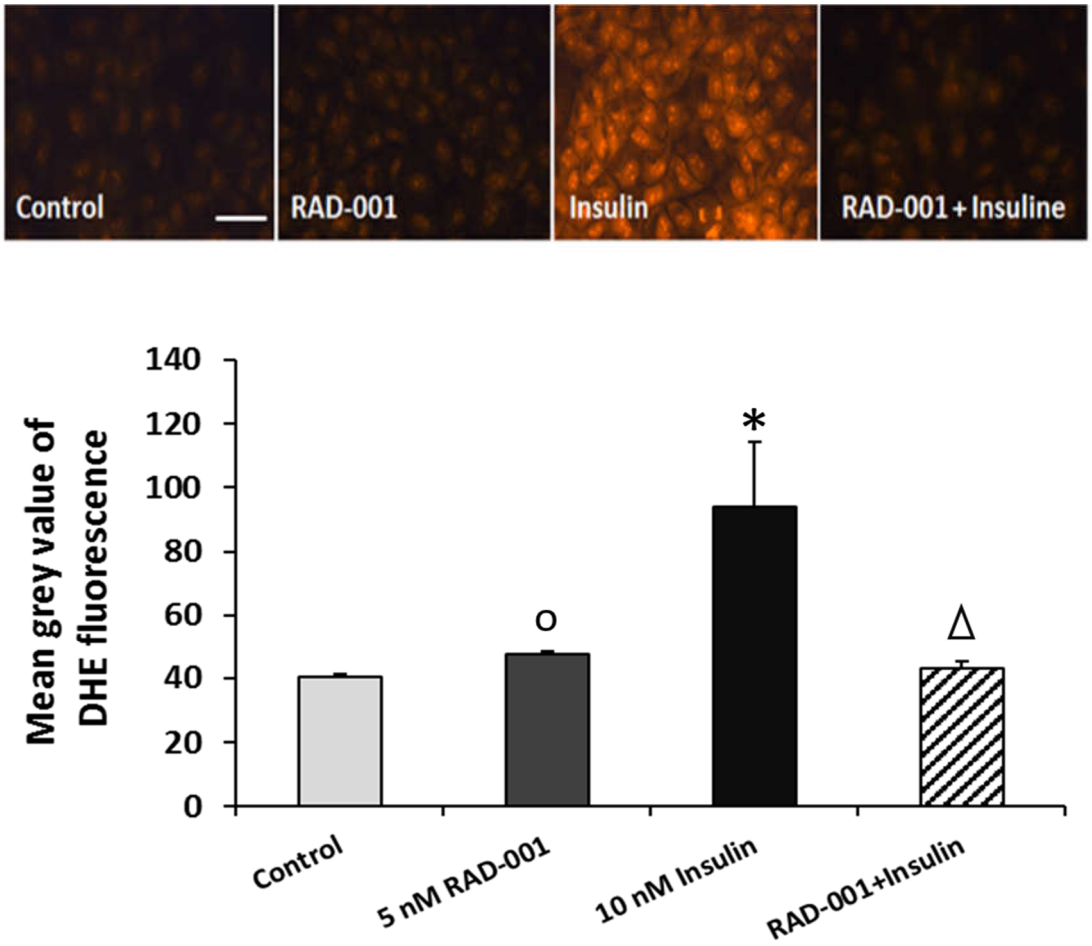


Figure 27. Microscopic detection of superoxide formation using the dye DHE in NRK cells treated for 15 min with 5 nM RAD-001 then addition of 10 nM insulin for 30 min in the presence of DHE. Quantification of DHE fluorescence was done by measuring the mean grey value of 200 cells using image j software. (*) Significantly different from control, (Δ) significantly different from insulin and (o) not significantly different from control. Scale bar 50 μm [64].

4.2.6 **Western blot analysis**

The quantification of the western blot analysis of phosphorylated AKT (p-AKT) in NRK cells showed that a significant increase in the amount of p-AKT protein was detected after the treatment with insulin compared to the control. RAD-001 increased significantly the amount of p-AKT, while resveratrol exerted small enhancement of p-AKT and no effect was observed for lovastatin alone. The lovastatin combination with insulin prevented the elevation of p-AKT induced by insulin. However, resveratrol did not exert any effect, while RAD-001 increased the amount of detectable p-AKT after

treatment with insulin but not significantly with respect to insulin alone (Figure 28) [64].

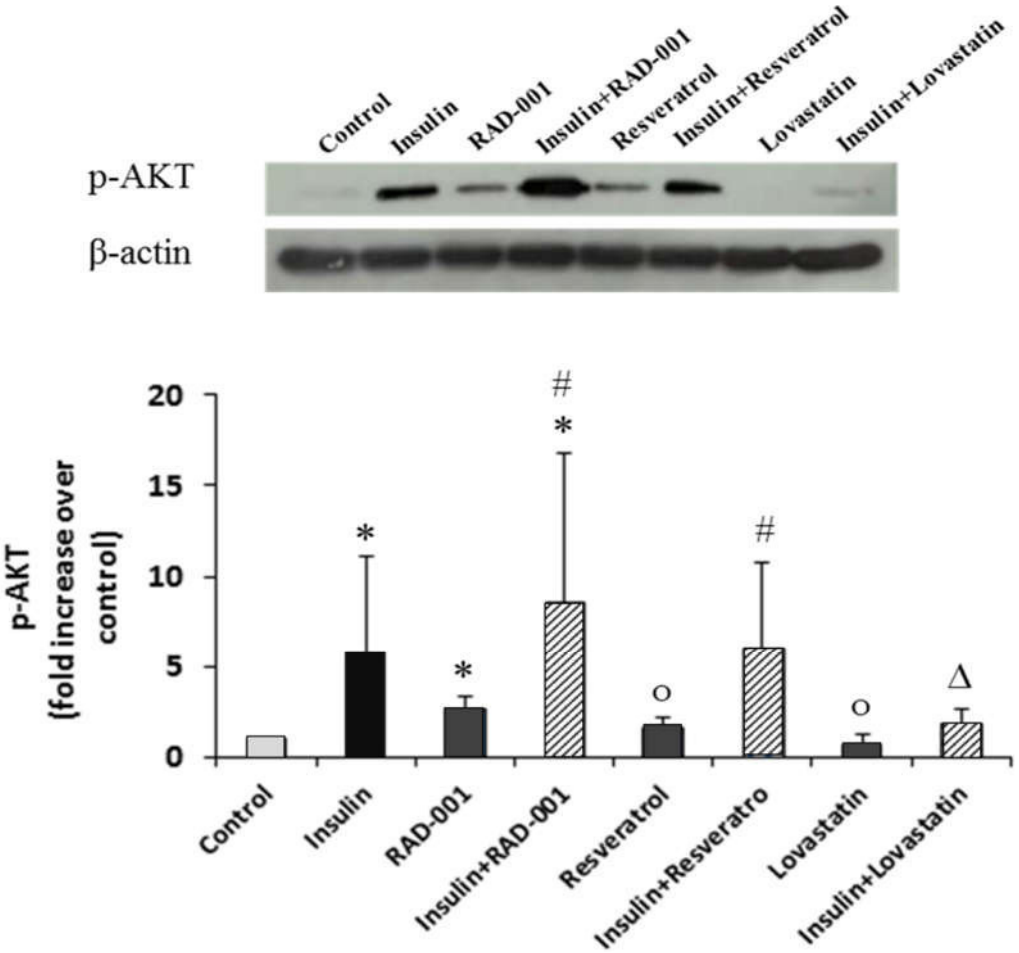


Figure 28. p-AKT level in NRK cells treated with 10 nM insulin, 5 nM RAD-001, 1 μM resveratrol and 10 nM lovastatin and their combination with insulin for 2 hours and analyzed by Western blotting. Blots from 3 independent experiments were used for quantification. (*) Significantly different from control, (Δ) significantly different from insulin, (o) not significantly different from control and (#) not significantly different from insulin [64].

4.3 Adrenaline, triiodothyronine, estradiol and progesterone hormones

4.3.1 The effect of hormones on HL-60 cells in vitro

Induction of genomic damage in HL-60 cells by adrenaline, T3, estradiol and progesterone, was investigated using two methods; the comet assay and the micronucleus frequency test

4.3.1.1 The influence of hormones on comet assay

The dose dependent experiments were conducted in HL-60 cells to verify which concentration (0.01-100 μM) is needed for hormones to exert their genotoxic effect. For estradiol, concentration which was described as effective in the literature was applied. The minimum concentration to induce a significant genotoxic response in the comet assay after 2 h treatment time was 100 nM for adrenaline (Figure 29), T3 (Figure 30), estradiol and progesterone (Figure 31). Therefore, this concentration was applied for further experiments.

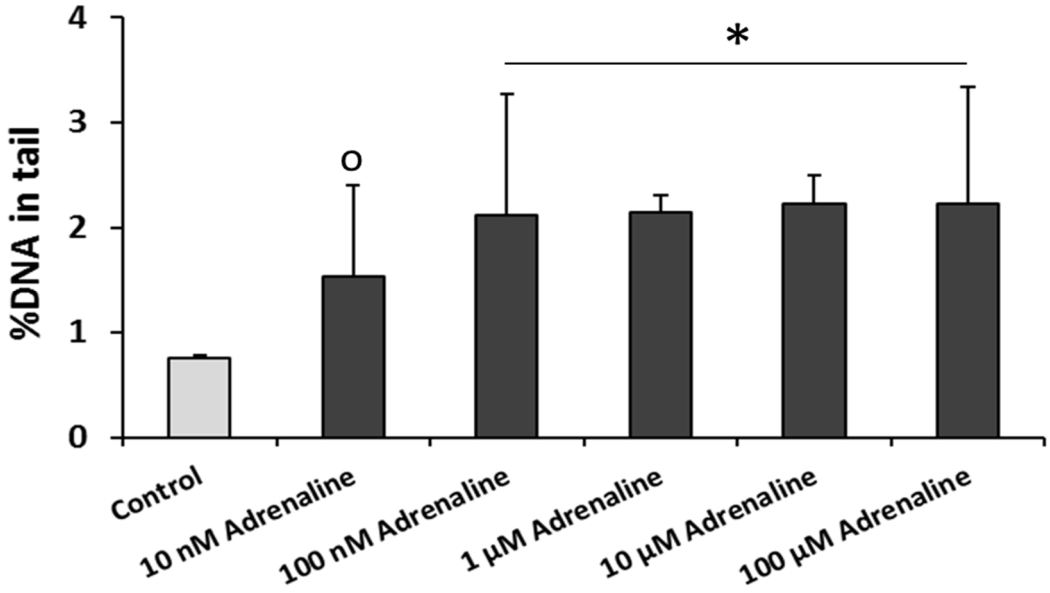


Figure 29. DNA damage (% DNA in tail) measured by comet assay analysis after treatment of HL-60 cells with the indicated concentrations of adrenalin for 2 hours. (*) Significantly different from control and (o) not significantly different from control.

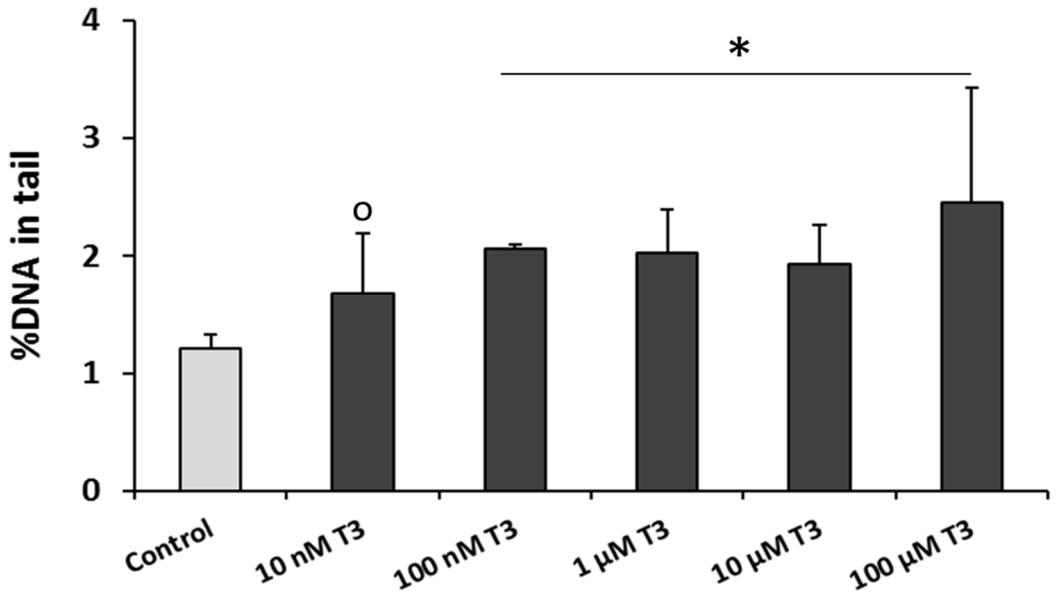


Figure 30. DNA damage (% DNA in tail) measured by comet assay analysis after treatment of HL-60 cells with the indicated concentrations of adrenalin for 2 hours. (*) Significantly different from control and (o) not significantly different from control.

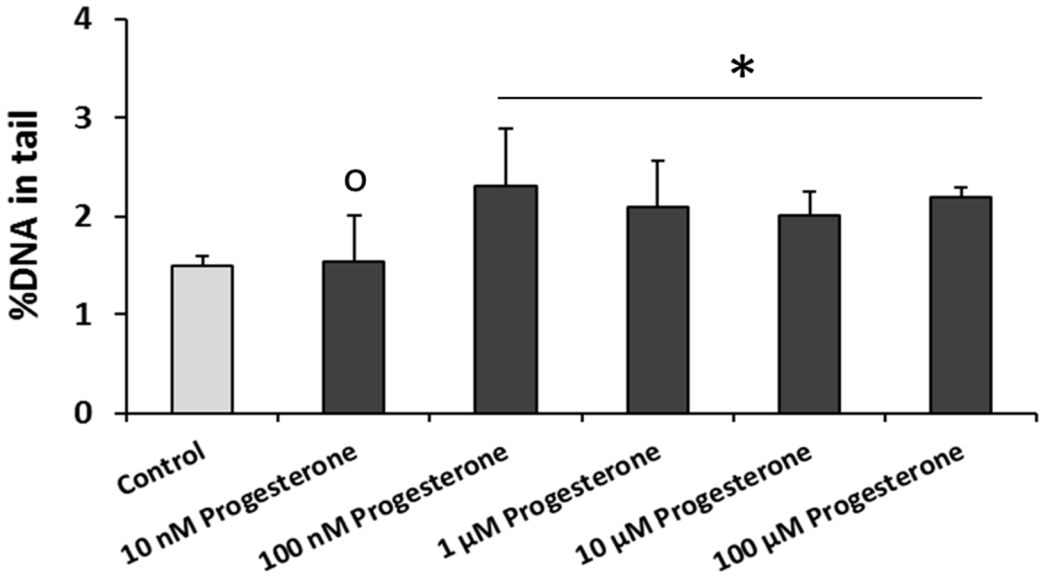


Figure 31. DNA damage (% DNA in tail) measured by comet assay analysis after treatment of HL-60 cells with the indicated concentrations of adrenalin for 2 hours. (*) Significantly different from control and (o) not significantly different from control.

As illustrated in Figure 32, 100 nM concentration for all tested hormones showed almost the same level of DNA damage compared to our positive control insulin after treatment of the cells for 2 hours. Additionally, the applied hormones had an ability to enhance the DNA damage significantly compared to the control.

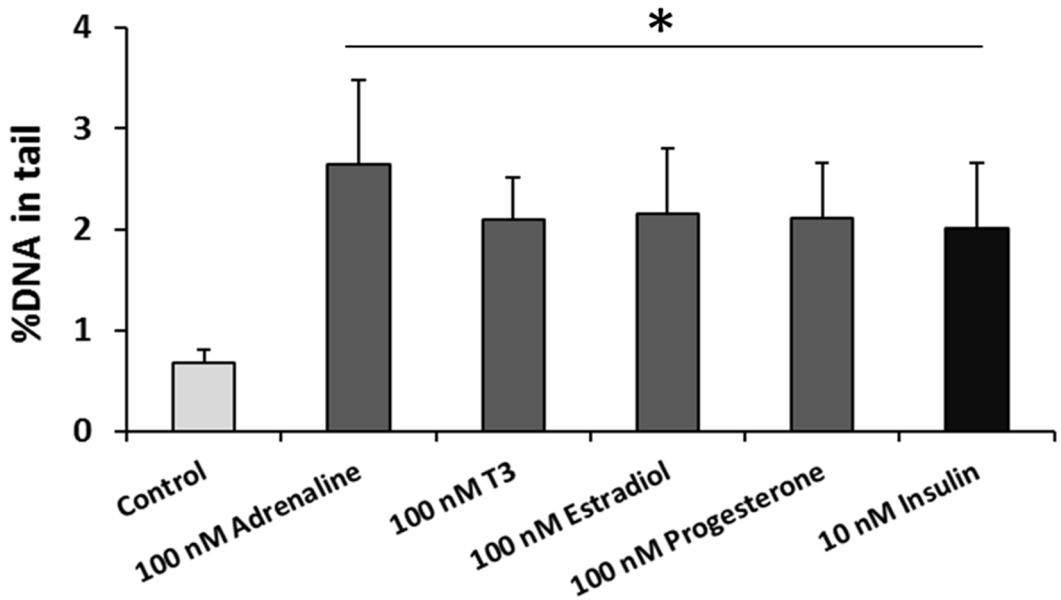


Figure 32. DNA damage (% DNA in tail) measured by comet assay after treatment of HL-60 cells with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (*) Significantly different from control.

4.3.1.2 **The influence of hormones on micronucleus formation**

The micronucleus induction assay was performed with 10 nM of insulin and 100 nM of other hormones in HL-60 cells for 4 h treatment time, then cytochalasin B was added for additional 20 hours without washing. The treatments of those hormones showed a significant increase in micronucleus formation compared to the control. Moreover, induction of proliferation index was detected in both estradiol and insulin after treatment conditions (Figure 33).

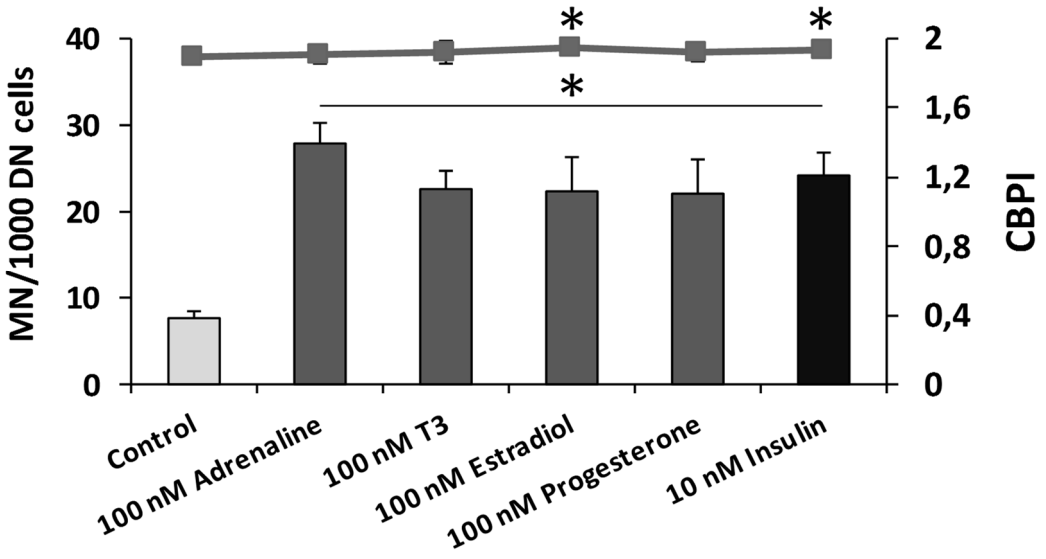


Figure 33. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in HL-60 cells treated with 100 nM adrenaline, T3, estradiol or progesterone, or 10 nM insulin for 24 h with cytochalasin B after stimulation for 44-48 hours with phytohemagglutinin (PHA). (*) Significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (–■–) and shown on the second y-axis.

4.3.2 **The influence of hormones on peripheral blood mononuclear cells (PBMC's) after mitotic stimulation with PHA**

This study included three main groups (non-pregnant, healthy pregnant and gestational diabetic). The non-pregnant group was subdivided into two subgroups according to the BMI to non-pregnant group with normal BMI (control) and non-pregnant group with higher BMI (obese). The characteristics of the study subjects is shown in Table 7.

Table 7. The characteristics of the study subjects. Groups were matched according to age and BMI. The group of non-pregnant women was subdivided into individuals with normal and with higher BMI. A significant ($P \leq 0.05$) difference in BMI occurred after this subdivision between these two groups and between these groups and healthy pregnant (only higher BMI) and GDM affected women. N = number of subjects per group.

Groups	N	Age (year)	BMI (kg/m^2)	Medications (%)	Mothers (%) [a]	Smokers (%)
Non-pregnant	32	31.69 ± 7.58	27.01 ± 10.38	L-Thyroxine (6.25) Blood pressure [c] (2.13)	51.72	21.88
Healthy pregnant	13	32.00 ± 3.83	25.32 ± 4.26 [b]	L-Thyroxine (23.08)	69.23	0
GDM	7	35.29 ± 4.03	31.04 ± 5.79 [b]	L-Thyroxine (28.57) Insulin (14.29) Amlodipine (14.29)	42.86	0
Non-pregnant with normal BMI (control)	26	31.38 ± 7.37	22.72 ± 2.65	L-Thyroxine (7.69)	50	23.08
Non-pregnant with higher BMI (obese)	6	33.00 ± 9.10	45.6 ± 11.06	L-Thyroxine (16.67) Blood pressure [c] (16.67)	16.67	16.67

(a) The percentage of women who had children (in pregnant women: prior to the current pregnancy).
(b) Determined between 13 and 38 weeks of pregnancy.
(c) Exact blood pressure medication is unknown.

4.3.2.1 **DNA damage in PBMCs of individuals of the non-pregnant group**

Two methods were used to detect the genomic damage; comet assay and micronucleus frequency test. Isolated PBMC's were stimulated with PHA, then incubated with 100 nM hormones, and 10 nM insulin. The DNA damage was elevated after treatment of cells of study participants of the control group with hormones in vitro compared to untreated PBMC's in the comet assay (Figure 34).

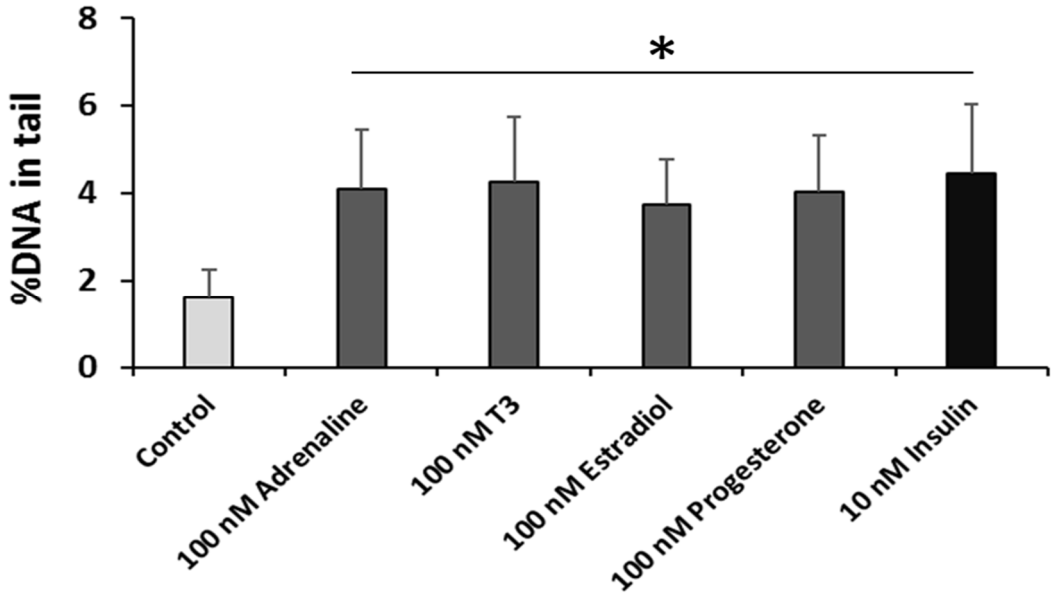


Figure 34. DNA damage (% DNA in tail) measured by comet assay after treatment of stimulated PBMC's in control group (N = 18) with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (*) Significantly different from control.

Moreover, adrenaline, T3, estradiol and progesterone induced a significant induction of micronucleus formation in PBMC's compared to the untreated control (Figure 35). Cell proliferation (CBPI) was unaffected.

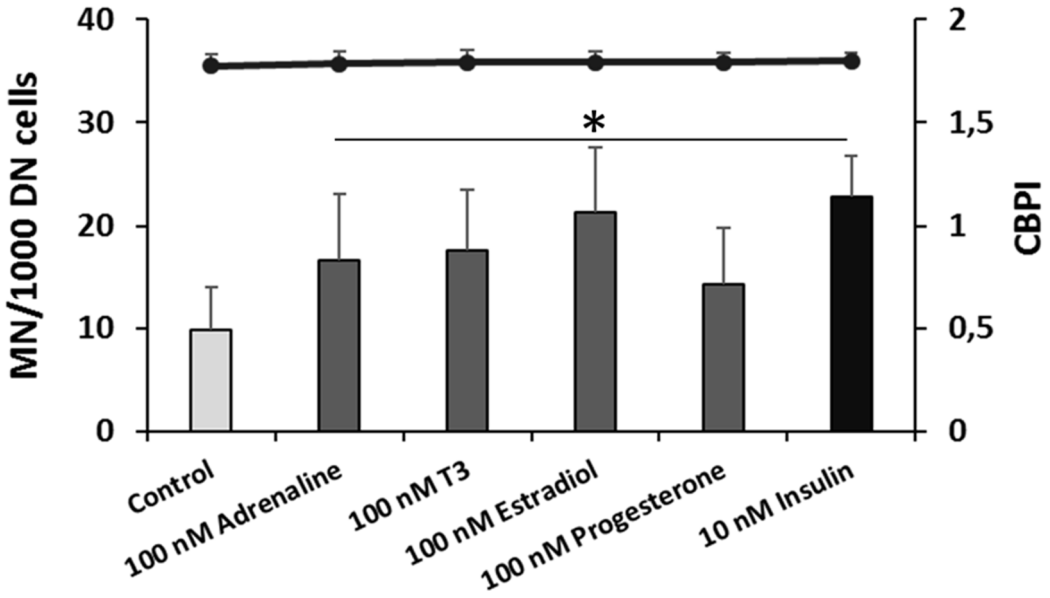


Figure 35. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in PBMC's for control group (N = 15) treated with 100 nM (adrenaline, T3, estradiol and progesterone), and 10 nM insulin for 24 h with cytochalasin B after stimulation for 44-48 hours with phytohemagglutinin (PHA). (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.2.2 **DNA damage in PBMCs of pregnant women**

We treated isolated PBMC's of pregnant women after mitotic stimulation with PHA with adrenaline, T3, estradiol, progesterone and insulin. All hormones showed a significant enhancement of DNA strand breaks compared to the control (Figure 36).

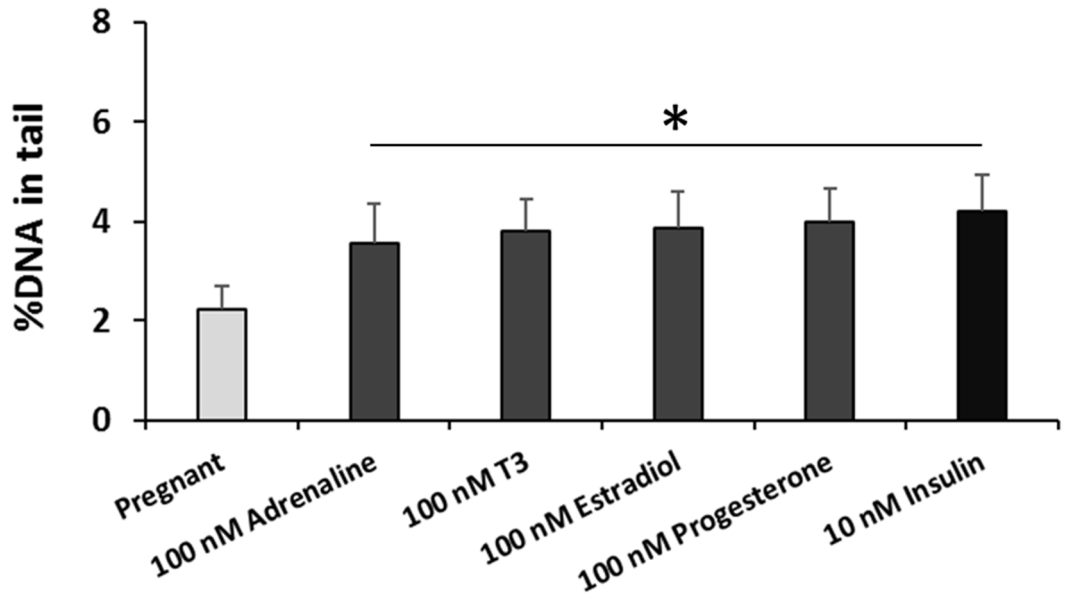


Figure 36. DNA damage (% DNA in tail) measured by comet assay after treatment of stimulated PBMC's in pregnant group (N = 10) with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (*) Significantly different from pregnant.

Additionally, micronucleus frequency analysis was performed. As presented in Figure 37, adrenaline did not show a significant induction of the number of micronucleus in 1000 double nucleated cells compared to the control. On the other hand, other hormones increased the genomic damage significantly. The cell proliferation (CBPI) was unaffected.

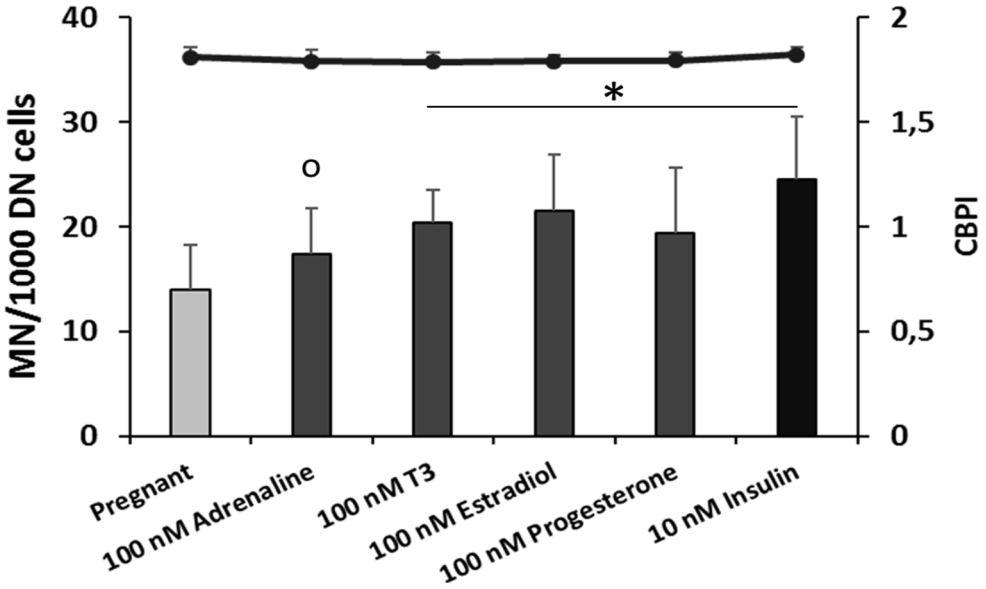


Figure 37. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in PBMC's for pregnant group (N = 10) treated with 100 nM (adrenaline, T3, estradiol and progesterone), and 10 nM insulin for 24 h with cytochalasin B after stimulation for 44-48 hours with phytohemagglutinin (PHA). (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.2.3 **DNA damage in PBMCs of women affected by gestational diabetes**

The PBMC's in GDM patients did not respond to treatment with hormones in vitro in the comet assay and the DNA damage was not significantly different from the control, except for 10 nM insulin, which caused increased DNA-damage (Figure 38).

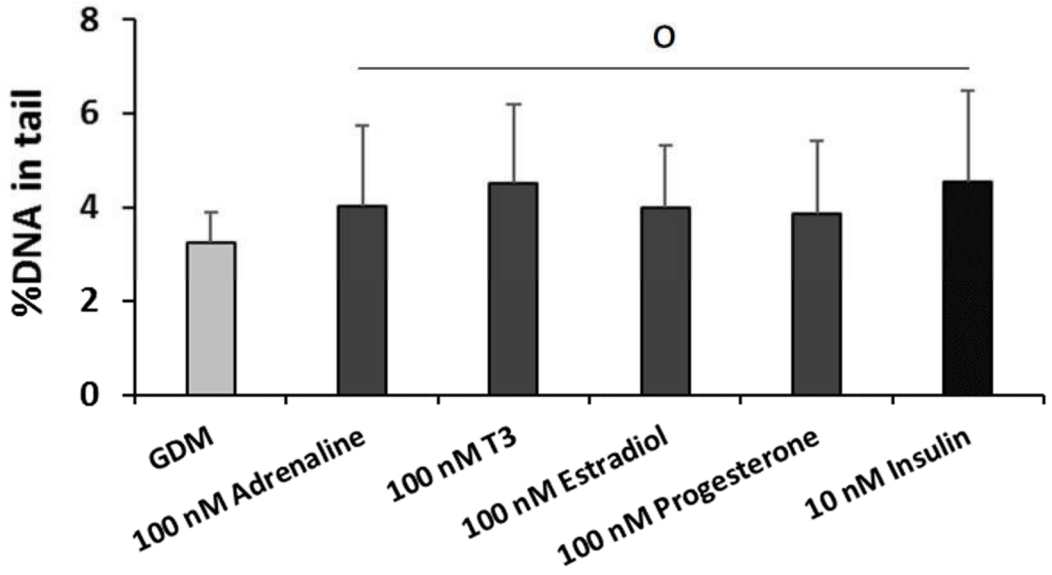


Figure 38. DNA damage (% DNA in tail) measured by comet assay after treatment of stimulated PBMC's in GDM group (N = 6) with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (o) Not significantly different from GDM.

Similar observations were made in the micronucleus frequency test, where the treated cells did not react to hormone treatment and the micronucleus formation was at the same level as in the untreated cells even with insulin (Figure 39). Again, the cell proliferation (CBPI) was unaffected.

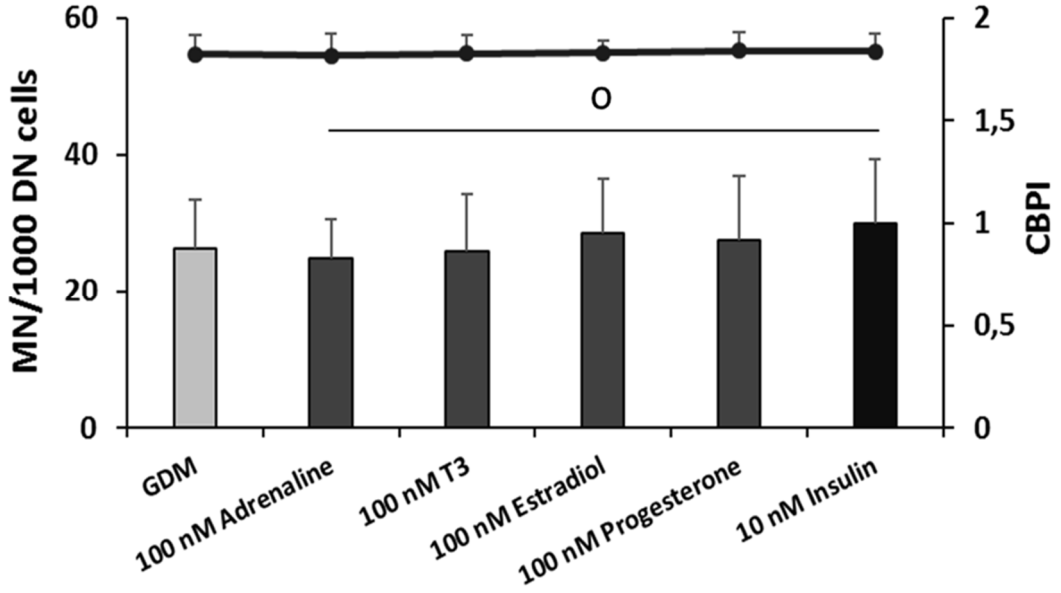


Figure 39. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in PBMC's for GDM group (N = 6) treated with 100 nM (adrenaline, T3, estradiol and progesterone), and 10 nM insulin for 24 h with cytochalasin B after stimulation for 44-48 hours with phytohemagglutinin (PHA). (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.2.4 **DNA damage in PBMCs of obese women**

In healthy obese women, the cells showed no significant induction of % DNA in tail after treatment with hormones in vitro (Figure 40).

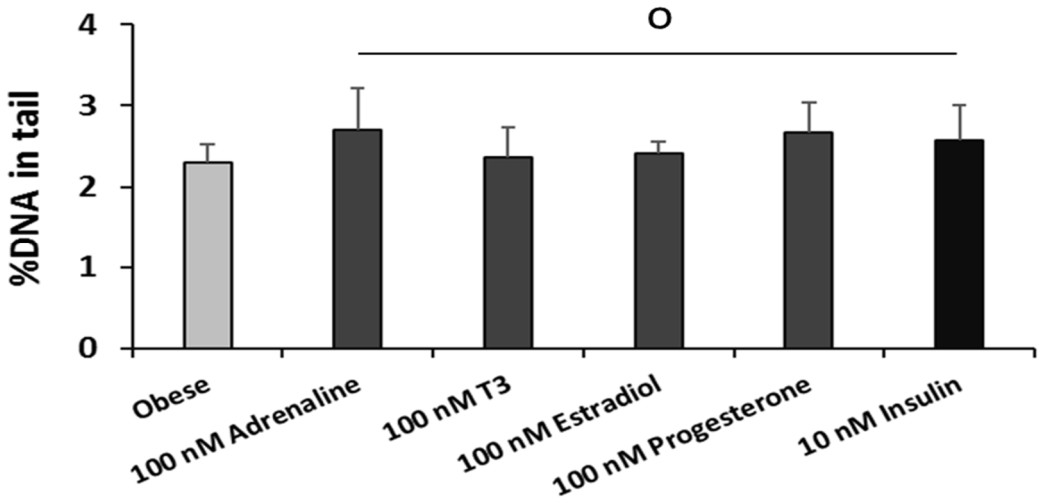


Figure 40. DNA damage (% DNA in tail) measured by comet assay after treatment of stimulated PBMC's in obese group (N = 6) with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (o) Not significantly different from obese.

Moreover, the genomic damage in micronucleus frequency test was also not affected by hormone treatment (Figure 41). Cell proliferation (CBPI) was unaffected. For easier comparison between groups, all results of the in vitro treatment of PBMCs are shown again in Figure 42 and Figure 43 for comet and micronucleus assays, respectively.

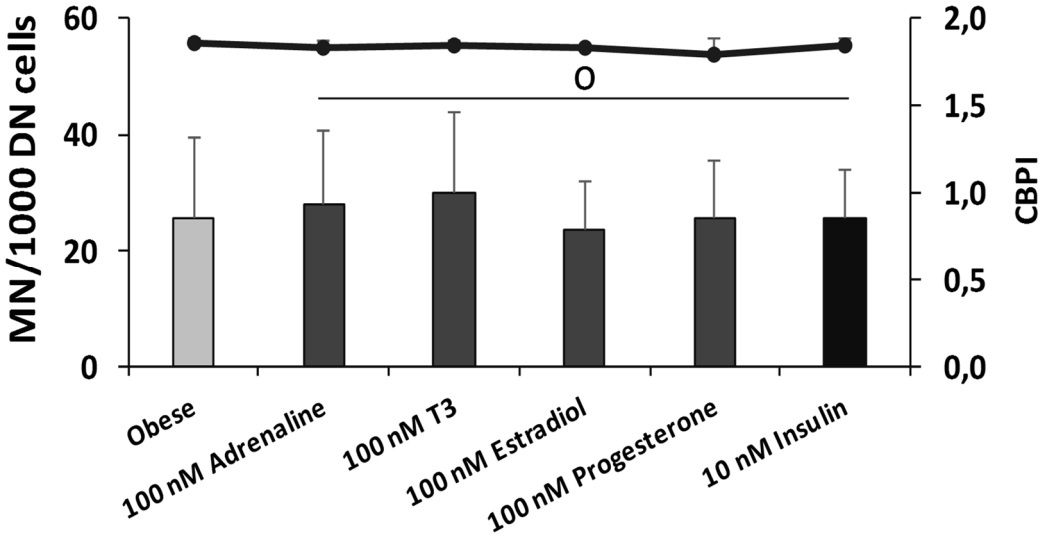


Figure 41. Micronucleus frequency (MN/1000 DN cells; DN: double nucleated cells) and proliferation index (CBPI) in PBMC's for obese group (N = 6) treated with 100 nM (adrenaline, T3, estradiol and progesterone), and 10 nM insulin for 24 h with cytochalasin B after stimulation for 44-48 hours with phytohemagglutinin (PHA). (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (■) and shown on the second y-axis.

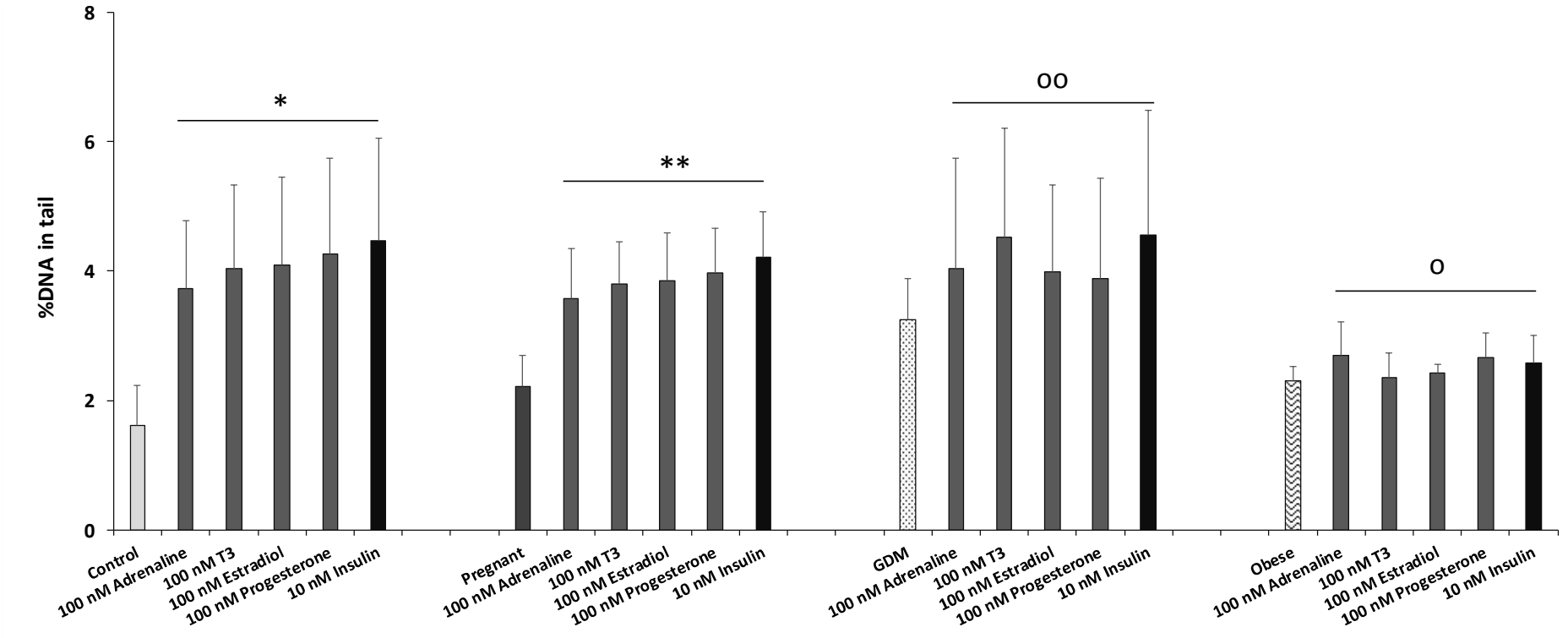


Figure 42. DNA damage (% DNA in tail) measured by comet assay after treatment of stimulated PBMC's in control, pregnant, GDM and obese women with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 2 hours. (*) Significantly different from control, (**) significantly different from pregnant, (oo) not significantly different from GDM and (o) not significantly different from obese.

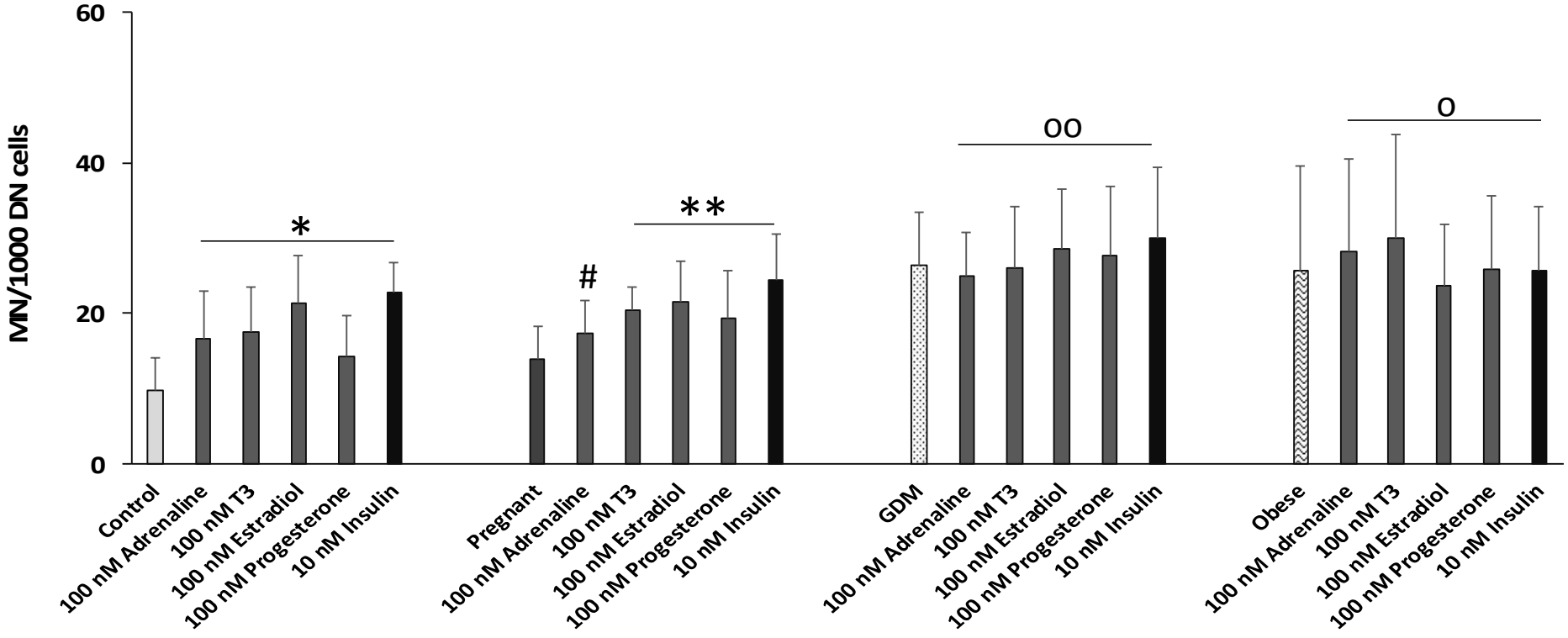


Figure 43. Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) after treatment of stimulated PBMC's in control, pregnant, GDM and obese women with 100 (adrenaline, T3, estradiol and progesterone) and 10 nM insulin for 24 hours with cytochalasin B. (*) Significantly different from control, (#) not significantly different from pregnant, (**) significantly different from pregnant, (oo) not significantly different from GDM and (o) not significantly different from obese.

4.3.3 **Comparison of genomic damage between study groups**

Next, we compared basal DNA damage levels between our study groups with three different method variations in the comet assay. We used whole blood, from which mononuclear cells are visible in comet assay analysis, isolated PBMCs and PBMC's which had been subjected to PHA-induced mitotic stimulation. With all samples, micronucleus analysis was also performed. However, only PHA-stimulated PBMC's are suitable for the micronucleus test, in which micronuclei in binucleated lymphocytes are detected.

4.3.3.1 **Comparison between control group with and without hormonal contraceptives**

The genomic damage was assessed in subjects who did not use hormonal contraceptives (HC) and who used HC. The results of the comet assay demonstrated that there was no statistically significant difference in DNA damage between the groups in whole blood-derived mononuclear cells, PBMC's and stimulated PBMC's (Figure 44).

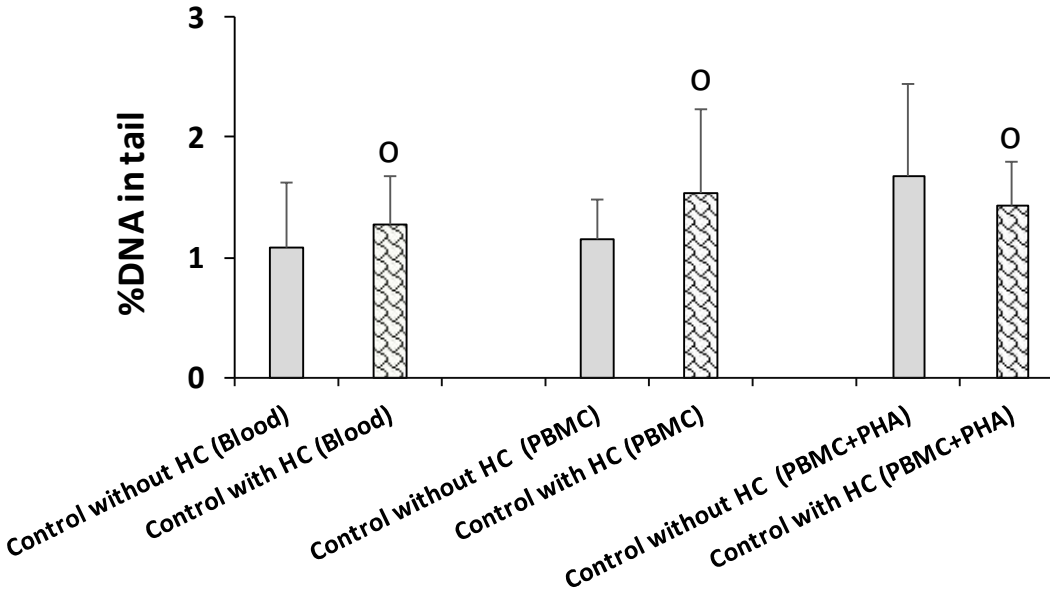


Figure 44. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: control without hormonal contraceptives (HC) (N = 11), control without hormonal contraceptives (HC) (N = 16), (II) PBMC's: control without hormonal contraceptives (N = 11), control without hormonal contraceptives (N = 16), (III) stimulated PBMC's with PHA for (42-44 h): control without hormonal contraceptives (N = 11), control without hormonal contraceptives (N = 8). (o) Not significantly different from control without HC.

For micronucleus assessment, only PHA-stimulated PBMCs can be employed and there was no significant difference in micronucleus frequency between the two tested groups (Figure 45). Cell proliferation (CBPI) was unaffected. Therefore, from here on we combined the two groups into one control group.

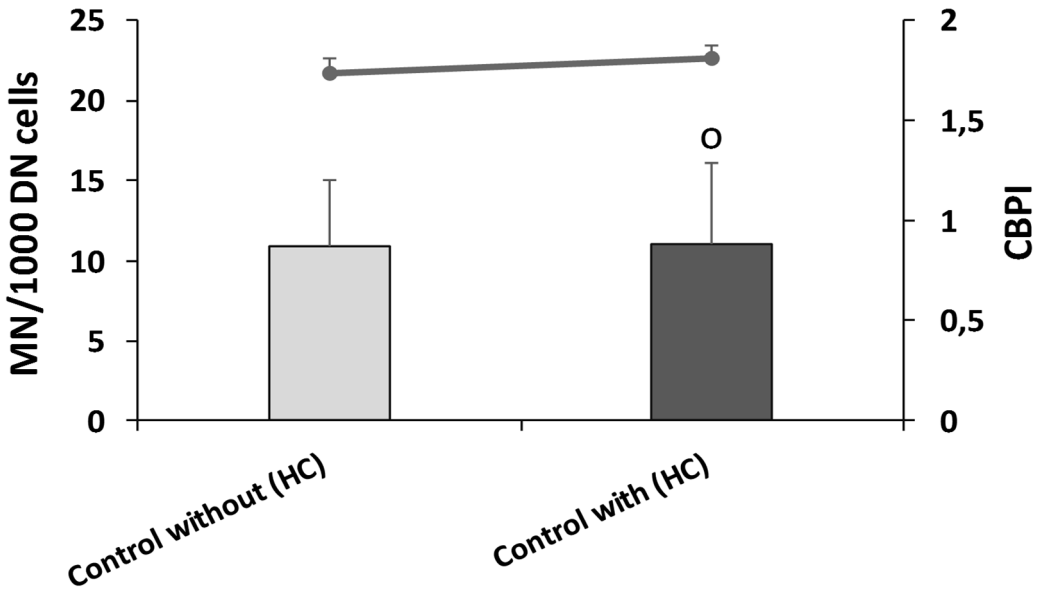


Figure 45**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) in PBMC's for control without hormonal contraceptives (HC) (N = 9) and control with hormonal contraceptives (HC) (N = 15) women after stimulation for (44-48) hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.3.2 **Comparison between control and pregnant groups**

To see if hormonal changes associated with pregnancy play a role in the detectable basal amount of DNA damage, we measured the levels of DNA damage in whole blood and PBMCs before and after mitotic stimulation with PHA. Pregnant women showed higher levels of DNA damage compared to controls in the comet assay in three approaches; whole blood-derived mononuclear cells, PBMC's without mitotic stimulation and with PHA-mediated mitotic stimulation (Figure 46).

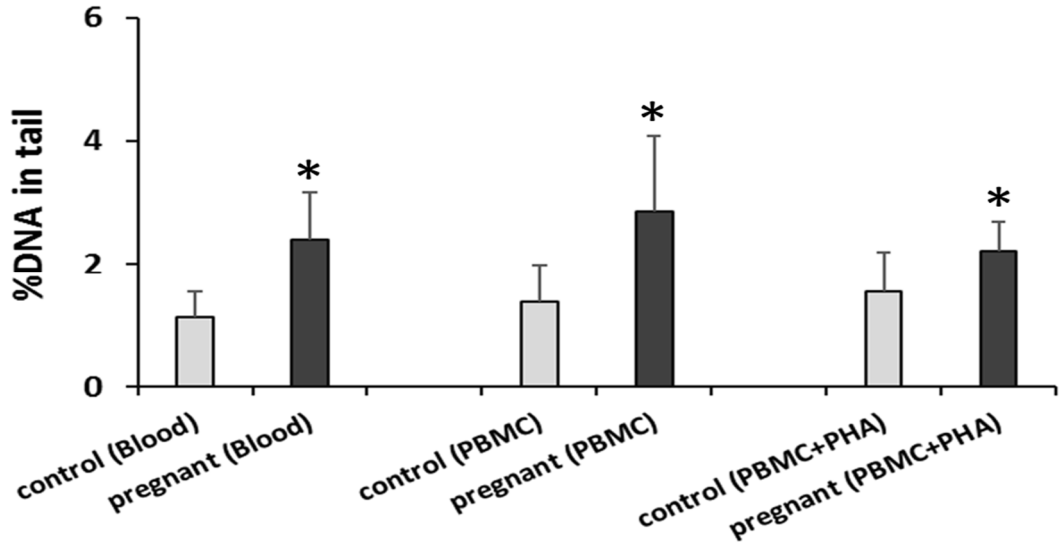


Figure 46. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: control (N = 26), pregnant (N = 13), (II) PBMC's: control (N = 26), pregnant (N = 13), (III) stimulated PBMC's with PHA for (42-44 h): control (N = 18), pregnant (N = 10). (*) Significantly different from control.

Furthermore, the slight induction of DNA damage observed in the pregnant group was not extended to induction of genomic damage in micronucleus frequency and the cell proliferation (CBPI) was unaffected. (Figure 47).

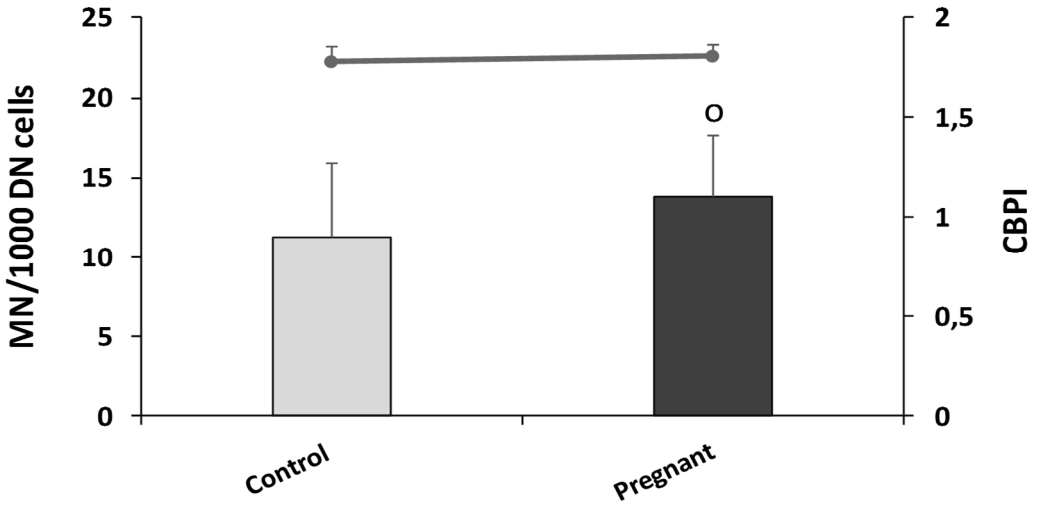


Figure 47**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) in PBMC's for control (N = 22) and pregnant (N = 13) women after stimulation for (44-48) hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (o) Not significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.3.3 **Comparison between pregnant and GDM groups**

For each examined group, the evaluations were obtained from comet assay and micronucleus frequency test. The results clearly showed that there was only a significant increase in DNA damage in PHA-stimulated PBMCs when GDM-affected pregnant women were compared with the healthy pregnant group by comet assay, (Figure 48).

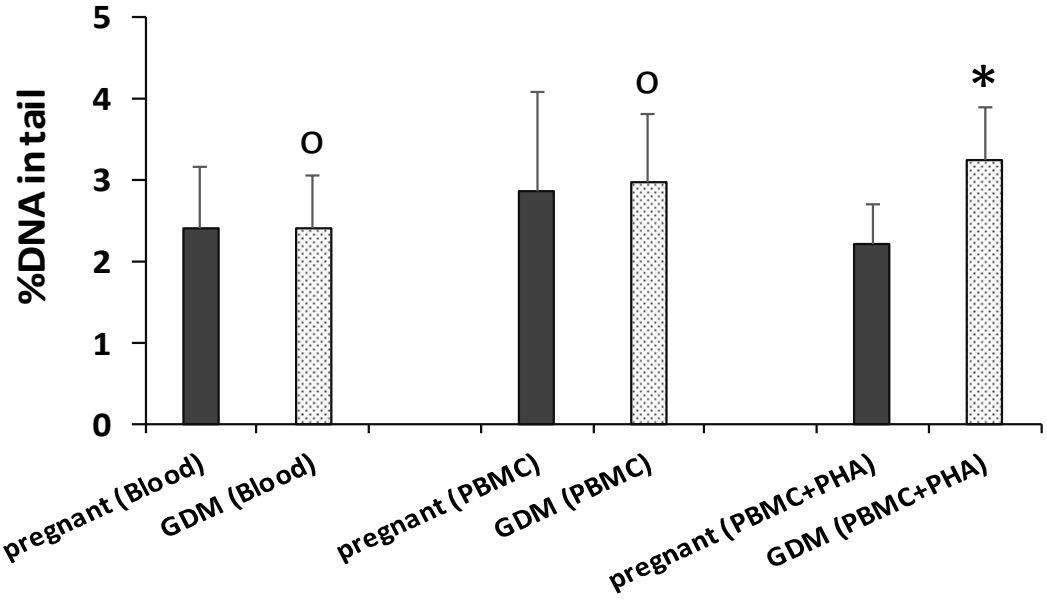


Figure 48. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: pregnant (N = 13), gestational diabetes mellitus (GDM) (N = 7), (II) PBMC's: pregnant (N = 13), GDM (N = 7), (III) stimulated PBMC's with PHA for (42-44 h): pregnant (N = 10), GDM (N = 6). (o) Not significantly different from pregnant, (*) significantly different from pregnant.

Additionally, the comparison of the results obtained from micronucleus frequency analysis showed that the GDM group exhibited increased formation of micronuclei compared to the healthy pregnant women (Figure 49). Cell proliferation (CBPI) was unaffected.

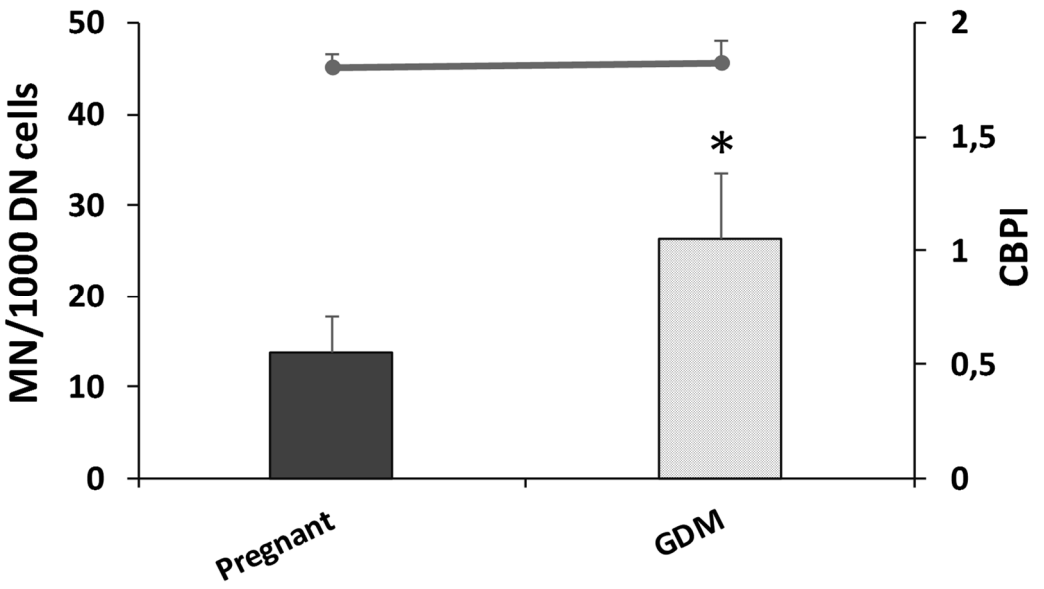


Figure 49**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) in PBMC's for pregnant (N = 13) and gestational diabetes mellitus (GDM) (N = 6) women after stimulation for (44-48) hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (*) Significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (–■–) and shown on the second y-axis.

4.3.3.4 **Comparison between control and obese groups**

Because there were several women with elevated BMI among our non-pregnant study participants, we studied the DNA damage in the obese subgroup (BMI ≥ 30) separately. Figure 50 illustrated that DNA damage was significantly enhanced in obese women under three conditions in the comet assay.

Moreover, an elevation was also noticed in in the micronucleus frequency test (Figure 51). Furthermore, the cell proliferation (CBPI) was significantly increased in obese women compared to control (non-obese) women. For easier comparison between groups, all results are represented again in Figure 52 and Figure 53 for comet and micronucleus assays, respectively.

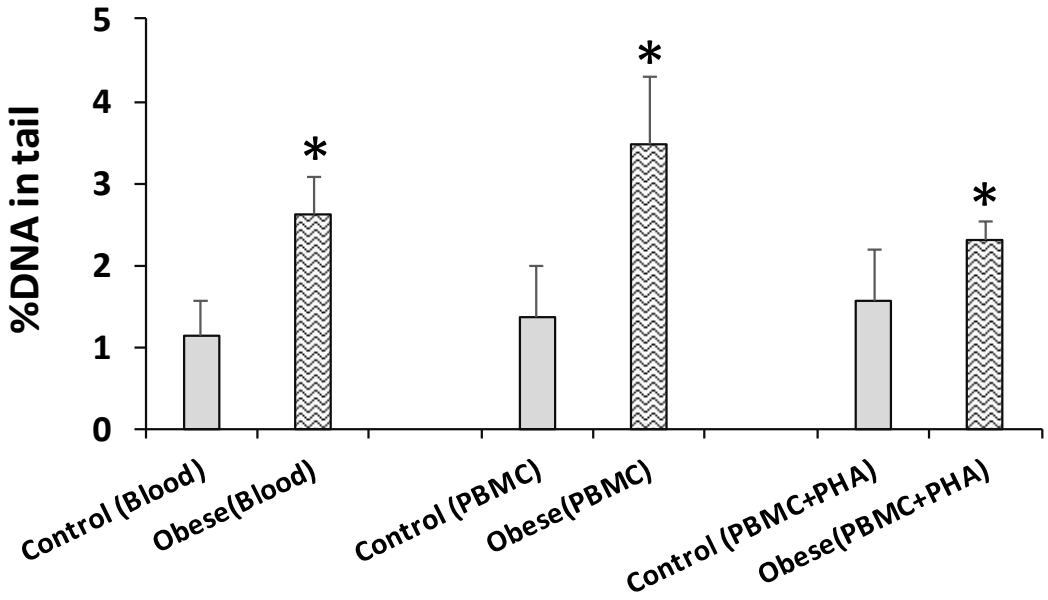


Figure 50. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: control (N = 26), obese (N = 6), (II) PBMC's: control (N = 26), obese (N = 6), (III) stimulated PBMC's with PHA for (42-44 h): control (N = 18), obese (N = 6). (*) Significantly different from obese.

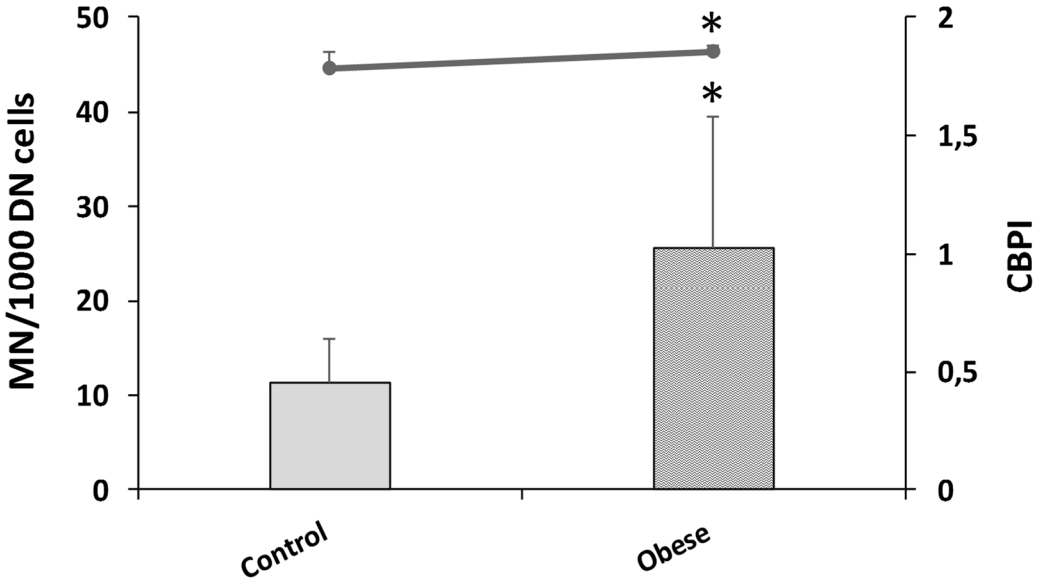


Figure 51**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) in PBMC's for control (N = 22) and obese (N = 6) women after stimulation for (44-48) hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (*) Significantly different from control. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

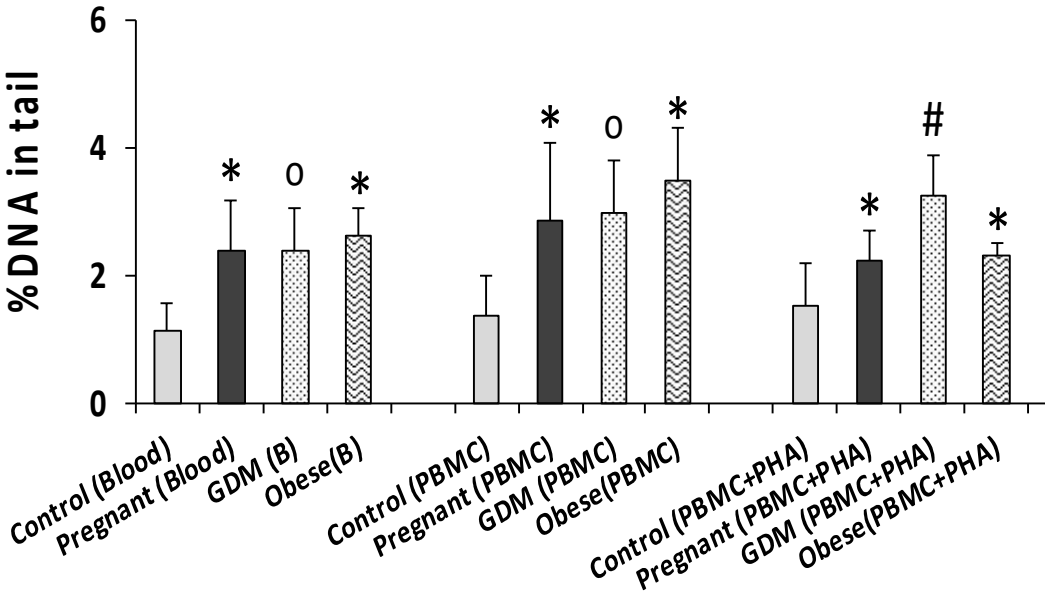


Figure 52. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: control (N = 26), pregnant (N = 13), GDM (N = 7) and obese (N = 6), (II) PBMC's: control (N = 26), pregnant (N = 13), GDM (N = 7) and obese (N = 6), and (III) stimulated PBMC's with PHA for (42-44 h): control (N = 18), pregnant (N = 10), GDM (N = 6) and obese (N = 6). (*) Significantly different from control, (o) not significantly different from control, (#) significantly different from pregnant.

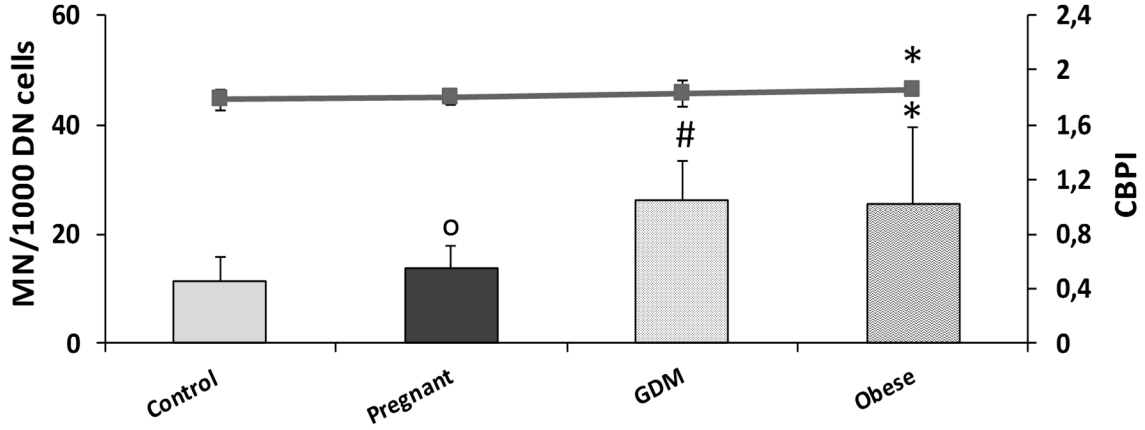


Figure 53**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) in PBMC's for control (N = 22), pregnant (N = 13), GDM (N = 6) and obese (N = 6) women after stimulation for (44-48) hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (*) Significantly different from control, (o) not significantly different from control, (#) significantly different from pregnant. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.3.5 **Comparison between pregnant women during pregnancy and after delivery**

Blood samples were collected again from pregnant women after delivery to evaluate the level of DNA damage. There was a significant reduction in DNA damage in both whole blood and unstimulated PBMC's (Figure 54) after pregnancy compared to during pregnancy. However, the slight reduction observed in stimulated PBMC's was not significant. The number of micronuclei in 1000 double nucleated cells (Figure 55) was reduced after delivery compared to during pregnancy. Cell proliferation (CBPI) was unaffected.

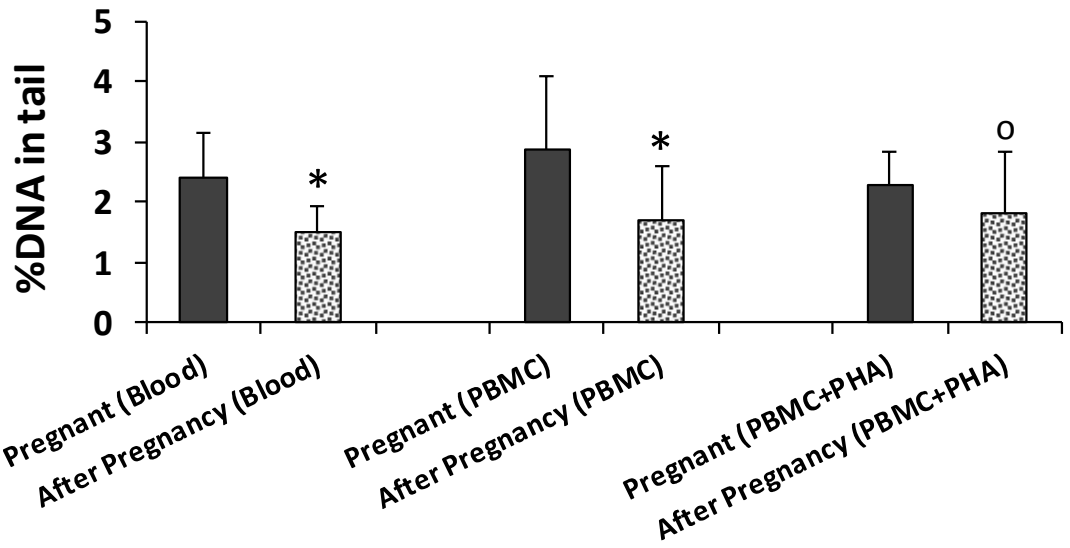


Figure 54. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: pregnant during (N = 13) and after pregnancy (N = 6), (II) PBMC's: pregnant during (N = 13) and after pregnancy (N = 6), and (III) stimulated PBMC's with PHA for (42-44 h): pregnant during (N = 11) and after pregnancy (N = 6). (*) Significantly different from pregnant, (o) not significantly different from pregnant.

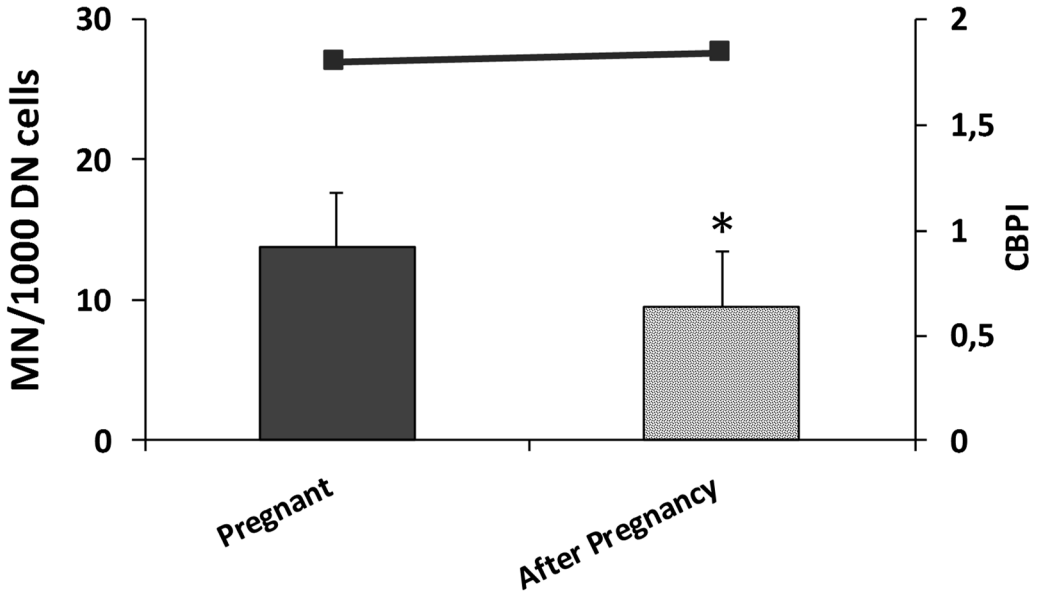


Figure 55**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) and proliferation index (CBPI) in PBMC's for pregnant women during (N = 13) and after pregnancy (N = 6) after stimulation for 44-48 hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (*) Significantly different from pregnant. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

4.3.3.6 **Comparison between gestational diabetes mellitus (GDM) during pregnancy and after delivery**

As shown in Figure 56, the DNA damage was decreased in GMD affected women after delivery as detected in the comet assay. While the reduction was not significant in mononuclear cells from whole blood, it was significant in unstimulated and stimulated PBMCs. The micronucleus frequency was also lower after pregnancy (Figure 57), while the cell proliferation (CBPI) was unaffected.

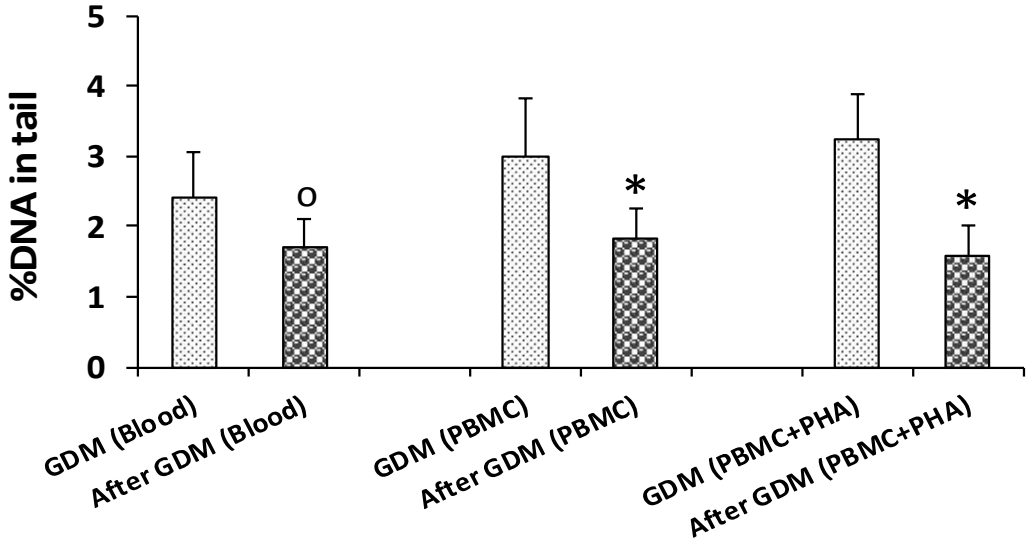


Figure 56. DNA damage (% DNA in tail) measured by comet assay among three conditions; (I) whole blood: gestational diabetes mellitus (GDM) during (N = 7) and after pregnancy (N = 7), (II) PBMC's: (GDM) during (N = 7) and after pregnancy (N = 7), and (III) stimulated PBMC's with PHA for (42-44 h): (GDM) during (N = 7) and after pregnancy (N = 6). (*) Significantly different from GDM, (o) not significantly different from GDM.

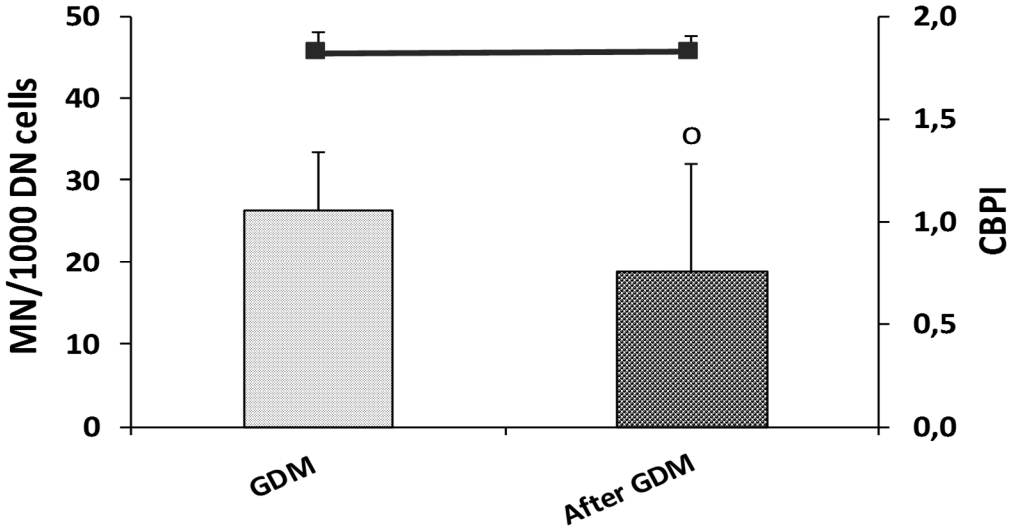


Figure 57**.** Micronucleus frequency (MN/1000 DN cells; DN = double nucleated cells) and proliferation index (CBPI) in PBMC's for gestational diabetes mellitus (GDM) women during (N = 7) and after GDM (N = 6) after stimulation for 44-48 hours with phytohemagglutinin (PHA). Harvest was after an additional 22 hours expression time. (o) Not significantly different from GDM. The cytokinesis-block proliferation index (CBPI) is represented as (-■-) and shown on the second y-axis.

5 Discussion

5.1 Modulation of insulin-induced genotoxicity in vitro

The phosphoinositide 3-kinase (PI3K) signaling pathway regulates a wide range of signaling transductions that play a significant role in many biological functions including cell proliferation, apoptosis, survival, differentiation, migration and cell growth [179-182]. The PI3K lipid kinases have been classified into three main classes (I, II and III) according to their sequence homology, substrate preference, expression and modes of regulation [84, 85]. Class I PI3Ks are heterodimeric lipid kinases subdivided into two subclasses including class IA and IB. For better understanding of the role of PI3K in the insulin signaling pathway in cells, specific PI3K inhibitors such as wortmannin and LY294002 were applied [183-186].

Thus, we have used three new PI3K inhibitors which are or have been under development of the pharmaceutical industry in addition to the well-known inhibitor wortmannin. The comet assay showed that the three new PI3K inhibitors led to significant reduction in DNA damage induced by insulin, but no significant differences between the three PI3K inhibitors or to wortmannin were observed.

Moreover, these inhibitors showed protective effects on insulin-mediated micronuclei as well as ROS formation in the same manner in NRK cells. In agreement with these results, Chaussade et al. showed that different isoform-specific class IA PI3K inhibitors all attenuating effects on insulin signaling in J774.2 macrophages [187]. Additionally, Chaussade et al. suggested that the ability of an isoform to be involved in insulin signaling is associated with its expression level in different cell types. In conclusion, we found that all PI3K inhibitors reduce the insulin-mediated ROS production and their cellular consequences.

Next, three particularly interesting natural and pharmacological compounds which have an ability to interfere with the insulin action pathway were selected. The three compounds resveratrol, lovastatin and RAD-001 have potential effects on insulin signaling through specific proposed mechanisms for these compounds [188-192].

Resveratrol is thought to decrease the phosphorylation of AKT, and the mammalian target of rapamycin (mTOR) in a concentration and time-dependent manner [193] and it has been found to stimulate glucose uptake in both skeletal muscle and adipose tissue [194, 195]. While lovastatin has a potentially inhibiting effect on ligand-induced receptor activation and its downstream signaling via the PI3K/AKT pathway and also by inhibition of NOX4 [196]. mTORC1 inhibitors such as RAD-001 (rapamycin analog) can play a significant role in the regulation of the PI3K/AKT/mTOR pathway through attenuation of mTORC1 downstream protein targets.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural polyphenolic compound that is mainly found in the skin of red grapes, has been described to enhance health, improve lifespan by 60% in short-lived fish [197] and act as sirtuin activator by mimicking caloric restriction [198]. Moreover, resveratrol has been investigated at a wide range of dose concentrations by Bhat et al. due to its interesting pharmacological properties [199]. Yet, resveratrol was also reported to exhibit bifunctional effects via several actions including both antioxidant and pro-oxidant effects depending on its concentration and exposure time [200].

Therefore, we investigated in the present work the protective properties of resveratrol on DNA damage induced after insulin treatment in rat kidney cells. Our results showed that resveratrol treatment decreased the oxidative stress, DNA damage and micronucleus formation in NRK cells in vitro. Additionally, the western blot analysis showed that the level of the phosphorylation of AKT which had been enhanced by insulin was not changed by resveratrol. However, resveratrol had the ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) in a cell-free system.

Our results are in agreement with reported work by Santos el. al who showed that resveratrol was able to protect the cellular DNA against oxidative damage, after a short treatment time of resveratrol (0.5 h; 10–250 μM resveratrol) under conditions of oxidative stress induced by hydrogen peroxide in C6 glioma cells [201]. However, slight DNA damage was induced in case of long treatment time with resveratrol (>6-48 h) time and dose-dependently [201].

In addition, Aydın et al. reported that the oxidative DNA damage in liver and kidney cells was reduced significantly in resveratrol-treated septic rats due to elevated levels of reduced glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GP_X) activities [202]. Overall, our findings suggest that resveratrol had no effect on the insulin signaling pathways under our treatment conditions but the protection of resveratrol against DNA damage might be through its antioxidant properties.

Lovastatin is a 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitor belonging to statin family. It can be isolated in low concentrations from natural sources such as red yeast rice [203] and oyster mushrooms [204]. It is mostly applied as a cholesterol-lowering agent in order to decrease the risk of cardiovascular disease in patients who suffer from hypercholesterolemia [205]. We found that lovastatin did not show intrinsic antioxidant activity in the FRAP assay but reduced the ROS overproduction enhanced by insulin in rat kidney cells as well as protected the cells from DNA damage and micronucleus formation after treatment with insulin.

Western blot analysis confirmed that p-AKT expression induced by insulin was significantly inhibited by lovastatin. Our findings are in agreement with Mcguire T. F., et al. who described that the association of PI3K with the IR/IRS-1 complex was disrupted in HIR rat-1 fibroblasts by lovastatin [190]. Therefore, it seems conceivable that the protective effects of lovastatin were caused through a signaling-mediated activity.

RAD-001 (Everolimus), a rapamycin analog, is a specific semi-synthetic mTOR inhibitor targeting the raptor/mTOR complex 1 (mTORC1). mTOR is a conserved regulator involved in different biofunctions such as proliferation, cell growth, survival and autophagy [206]. Different stimuli including nutrients and growth factors regulate the activation of mTORC1 by different mechanisms [207]. Moreover, negative feedback loops are induced via the mTORC1 downstream S6K that inhibit the activation of the PI3K-AKT signaling pathway. Therefore, the inhibition of the negative feedback loop promotes AKT phosphorylation [208].

As previously described, insulin stimulates NRK cells immediately to upregulate the intracellular kinase cascades which are involved in the activation of the PI3K/AKT signaling pathway, as well as activation of NADPH oxidase and mitochondria and resulting in cellular ROS generation [209]. Reported studies described that the overactivation of mTOR showed an increase in mitochondrial biogenesis and more DNA damage due to accumulation of reactive oxygen species [207, 210].

Among the main cellular sources of ROS production in mitochondria, complex I and complex III are the major mediators of ROS due to the electron leakage from the electron transport chain [211]. Therefore, inhibition of the raptor/mTOR complex by RAD-001 may play a key role in mitochondrial activity via the reduction of the activity of mitochondrial complex I [212].

Our results are in agreement with previous ideas, while in our investigation, no increase in ROS formation after addition of RAD-001 was obtained. Moreover, the pretreatment of the cells with RAD-001 blocked the ROS induced by insulin under the experimental conditions. Additionally, the pretreatment of the kidney cells with the mTORC1 inhibitor RAD-001 before the insulin treatment also suppressed the DNA and genomic damage stimulated by insulin, but it did not protect the kidney cells completely. Furthermore, the western blot analysis showed that the amount of p-AKT was enhanced by RAD-001 alone as well as by the combination of RAD-001 with insulin.

The enhancement in p-AKT obtained from the combination of RAD-001 with insulin was not significant compared to insulin alone. Our results are in agreement with Nacarelli et al. who reported that the pretreatment of human diploid fibroblasts with rapamycin inhibitor increased the phosphorylation of AKT and decreased the level of mitochondrial ROS stimulated by ethidium bromide [213]. In addition, Miwa et al. proposed that the function of telomerase protein TERT in mitochondria might be improved via the inhibition of mTOR signaling pathway by rapamycin to reduce the mitochondrial ROS [214]. Overall, oxidative stress can be decreased via inhibition of mTORC1 complex by RAD-001 protecting the cellular components against damage and that could be due to the impact of mitochondria through insulin signaling pathway.

In conclusion, resveratrol, lovastatin and RAD-001 showed protective activities and reduced the genomic damage induced by insulin in rat kidney cells in vitro Moreover, a combination of these interesting compounds with insulin may be of potential relevance to support T2DM therapy and prevent co-morbid diseases such as cancer. This can be achieved through several molecular activity principles, which may provide greater chances for the development of medical treatment strategies for patients.

5.2 Genomic damage in gestational diabetes mellitus (GDM)

Different types of diabetes mellitus can occur in pregnant women, including type I, type II diabetes mellitus and gestational diabetes mellitus (GDM) [215, 216]. GDM is known as an elevation of glucose level in the blood during pregnancy without previously diagnosed diabetes. The overall percentage of GDM affected women in Germany is about 13.2%. Among these percentage, the prevalence of GDM affected women was observed to be increased with maternal age around 45 and older [217]. Moreover, GDM can also be associated with several outcomes not only related to mother, but also to the child [217]. One of the long-term complications as a consequence of GDM could be rising the risk of type II diabetes mellitus.

During pregnancy, women experience changes in the levels of hormones such as estradiol, progesterone and others. This can mediate insulin resistance and cause an elevation of the response of pancreatic beta cells which is important for the development of the fetus [216]. Moreover, the level of oxidative stress increases due to the mitochondria-rich placenta condition during pregnancy [218]. As mentioned before, a high insulin level in blood can be associated with oxidative stress.

Thus, GDM is thought to be associated with high levels of oxidative stress either due to the elevation of reactive oxygen species or an impaired antioxidant defense system [219]. This study was conducted to investigate the correlation between gestational diabetes mellitus and genomic damage derived from human blood. Previous studies regarding female hormones or GDM investigated the damage in PBMC's, plasma or saliva and they also used other cytogenetic assays [220, 221]. Yet, there have been no studies using comet assay and micronucleus analysis in the same way as we did here. We applied three conditions, namely whole blood-derived mononuclear cells (comet assay), PBMC's (comet assay) and stimulated PBMC's (comet assay and micronucleus analysis). The study groups consisted of women with or without the use of hormonal contraceptives, pregnant women, and GDM-affected women. Among the non-pregnant women, a subgroup of overweight women could be identified.

A first point to discuss is the potential role of hormonal contraceptives. Trebuňová et al. did not observe genotoxic effects by high resolution melt analysis in women using oral contraceptives [221]. However, Biri el al. noticed a significant elevation of the mean damage values in sister chromatid exchanges (SCE) and comet assay in women using hormonal contraceptives [220]. Our results demonstrated that there was no statistically significant difference between women using or not using hormonal contraceptives in any of the methods or variations. Thus, for our further comparisons, we merged these two groups into one group described as control group.

The second point, which was the major focus of our study, was whether GDM has any influence on the genomic damage level. Earlier studies reported that significant induction of DNA damage in GDM and pregnant women was observed [222-224]. Moreover, other studies showed that GDM and pregnant women showed a moderate increase in structural chromosome aberrations with no change to be considered [225].

In our study, a significant increase in DNA damage in healthy pregnant women compared to controls in whole blood, PBMC's before and after stimulation, analyzed by comet assay was observed. However, the micronucleus analysis did not show enhanced genomic damage in healthy pregnant women. The high level of progesterone during pregnancy which is around 10-fold more than in non-pregnant women could be the causative agent for protecting via inhibition of ROS production by transcriptional regulating of the expression of myeloperoxidase and NADPH [226].

On the other hand, our data regarding GDM subjects evidence significant elevation of DNA damage compared with healthy pregnant women in stimulated PBMC's in both comet assay and micronucleus frequency test. Some support for our findings may be provided by two studies which noticed elevation of oxidative DNA damage in gestational diabetes pregnant women [223, 224, 227]. Moreover, it has been found that the antioxidant defense system is also impaired in GDM women [224, 228]. Fortunately, our results indicated that the effect of GDM disappears after delivery and we observed a decrease in the level of DNA strand breaks in the comet assay at two months after birth. We also observed some reduction of the micronucleus frequency, however not the same extent. The reason may be that damage in the comet assay is

repairable and can disappear even at the single cell level within a short time. On the other hand, micronuclei or the chromosomal damage from which they are formed persist in cells and therefore the damage level decreases only if the lymphocyte population is replaced by fresh lymphocytes. It has been estimated that the half-life of an existing elevated micronucleus frequency may be in the order of several months [229]. Since our follow up within this study was at 2 months after birth, a longer observation than the duration of our study period may show more micronucleus frequency reduction. It would be important to study this with a larger number of GDM-affected women and over a longer time period. It would also be important to see whether nutritional recommendations, such as high vitamin B or folate intake [230] or a diet rich in resveratrol, or medication such as lovastatin might aid in the further reduction of the micronucleus frequency. Nothing is known about a possibly enhanced future cancer risk of women affected by GDM, but they are at risk for the later occurrence of T2DM [231-233] which in turn is associated with an elevated cancer risk.

During pregnancy, many physiological changes and endocrine alterations can occur [234]. Hormone dysfunction during pregnancy can cause several problems on mother and fetus and effects are serious if left untreated [234]. Additionally, several issues may require hormonal therapy including; thyroid disorder resulting in hypothyroidism or hyperthyroidism [235-237] and signs of threatened abortion or preterm birth [238-240]. Moreover, hormonal levels can be influenced by stress during pregnancy or lifestyle factors.

With this in mind, we decided to evaluate the influence of some hormones on cells from controls, pregnant and GDM-affected women to see whether their pre-existing hormonal differences (such as elevated insulin levels in GDM) have any influence on the sensitivity of the cells in vitro.

With HL-60 cells, which we first used as a model for blood-derived cells, we found elevated DNA damage after treatment with estradiol, progesterone, adrenaline and T3. When we treated isolated PBMC's of the control group, we also found an increase with all hormones in the comet-assay as well as the micronucleus test. Our findings are in agreement with Zambrano et al. who showed that treatment of mice with T3 increased

liver and kidney oxidative DNA damage [121]. Additionally, Okoh et al. clarified that estrogen-induced 8-hydroxy-guanine (8-OHdG) and increased the ROS production, therefore enhanced the DNA damage in the MCF-7 cell line [241]. Moreover, Radaković et al. concluded that adrenaline plays a role in the induction of DNA damage due to reactive oxygen species in isolated lymphocytes but this damage can be repaired within the first four hours after treatment[242]. However, for progesterone protective effects may be expected, since inhibition of ROS production by transcriptional regulating of the expression of myeloperoxidase and NADPH [226] had been found. However, we detected increased DNA damage after in vitro treatment with progesterone. This could be explained by observations that progesterone inhibited apoptosis, thus allowing cells with damaged DNA to survive [243]. In addition, progesterone exhibits a biphasic effect on cell proliferation in vitro in which progesterone initially accelerates the proliferation in the cell-cycle through increased S-phase entry at 18 hours followed by mediating of cyclins (G1/S and G2), p21 and CDK2 for 24 to 48 h i.e. 1–3 cell cycles. Next, the cell-cycle arrests at the G1/S by cell-growth inhibition through p27 upregulation[244]. Therefore, it was suggested that progesterone exerts both proliferative and inhibitory effects[244]. In cells from our other study group, GDM-affected women, there was no significant increase due to in vitro hormone treatment.

Recently, many studies reported that obesity in childhood or elevation of maternal BMI can be a factor of risk prevalence in developing GDM [245, 246]. Furthermore, elevated body weight has also been described to be associated with alterations in hormone levels [176, 247-253]. Additionally, obese individuals are known to have elevated DNA damage due to the increase of reactive oxygen specious [254, 255]. For that reason, we treated the stimulated PBMC's of obese women (non-pregnant; BMI $\geq$ 30) with several hormones.

Our results showed higher DNA damage in obese women in the values obtained from three conditions (whole blood, PBMC's before and after stimulation) than in control women with normal weight (BMI $\leq$ 25) in comet assay and micronucleus frequency test. That finding is in agreement with Bankoglu et al. who presented that weight loss either by gastric bypass surgery or caloric restriction reduced the elevation of oxidative

stress and genomic damage that was associated in obese Zucker (fa/fa) rats [256]. However, the stimulated PBMC's of the obese women were not affected by the in vitro hormone treatment in both comet assay analysis and micronucleus formation.

In conclusion, while basal DNA damage was elevated in pregnant, obese and GDM-affected women, their PBMC's did not show additional DNA damage after in vitro treatment with hormones. However, PBMCs of normal weight non-pregnant women and HL-60 cells exhibited elevated DNA damage after treatment with each of the tested hormones. One hypothesis may be that through hormonal activation only a certain maximal induction of DNA damage can be achieved. In those groups that experienced the induction through in vivo elevated hormonal levels, the maximal induction may already be present as basal level and no further induction could then be achieved by in vitro treatment. However, this has to be investigated in further detail, elucidating the mechanisms for induction of DNA damage for each of the hormones in vitro and identifying the causes of elevated basal values in vivo, which may depend on other factors in addition to the investigated hormones. If it holds true, that no further increase beyond a certain maximal level can be achieved, additional hormonal influences during a pregnancy with GDM or in obesity may not lead to a (further) increase of cancer risk.

6 Summary

Diabetes mellitus is a global health problem, where the risk of diabetes increases rapidly due to the lifestyle changes. Patients with type II diabetes have many complications with increased risk of morbidity and mortality. High levels of insulin may lead to DNA oxidation and damage. Several studies proposed that hyperinsulinemia may be an important risk factor for various types of cancer. To investigate insulin signaling pathway inducing oxidative stress and genomic damage, pharmaceutical and natural compounds which can interfere with the insulin pathway including PI3K inhibitors, resveratrol, lovastatin, and RAD-001 were selected due to their beneficial effects against metabolic disorder. Thus, the anti-genotoxic potential of these compounds regarding insulin-mediated oxidative stress were investigated in normal rat kidney cells in vitro. Our compounds showed protective effect against genotoxic damage and significantly decreased reactive oxygen specious after treatment of cells with insulin with different mechanisms of protection between the compounds. Thus, these compounds may be attractive candidates for future support of diabetes mellitus therapy.

Next, we explored the link between gestational diabetes mellitus and genomic damage in cells derived from human blood. Moreover, we investigated the influence of estradiol, progesterone, adrenaline and triiodothyronine on insulin-induced genomic damage in vitro. First, we studied the effect of these hormones in human promyelocytic leukemia cells and next ex vivo with non-stimulated and stimulated peripheral blood mononuclear cells. In parallel, we also measured the basal genomic damage using three conditions (whole blood, non-stimulated and stimulated peripheral blood mononuclear cells) in a small patient study including non-pregnant controls with/without hormonal contraceptives, with a subgroup of obese women, pregnant women, and gestational diabetes affected women. A second-time point after delivery was also applied for analysis of the blood samples. Our results showed that GDM subjects and obese individuals exhibited higher basal DNA damage compared to lower weight non-pregnant or healthy pregnant women in stimulated peripheral blood mononuclear cells in both comet and micronucleus assays. On the other hand, the DNA damage in GDM women had decreased at two months after birth. Moreover, the applied hormones also showed an influence in vitro in the enhancement of the genomic damage in cells of the

control and pregnant groups but this damage did not exceed the damage which existed in obese and gestational diabetes mellitus patients with high level of genomic damage. In conclusion, insulin can induce genomic damage in cultured cells, which can be modulated by pharmaceutical and naturals substances. This may be for future use in the protection of diabetic patients, who suffer from hyperinsulinemia during certain disease stages. A particular form of diabetes, GDM, was shown to lead to elevated DNA damage in affected women, which is reduced again after delivery. Cells of affected women do not show an enhanced, but rather a reduced sensitivity for further DNA damage induction by hormonal treatment in vitro. A potential reason may be an existence of a maximally inducible damage by hormonal influences.

7 Zusammenfassung

Diabetes mellitus stellt eine globales Gesundheitsproblem dar, das aufgrund der sich ändernden Lebensführung rapide ansteigt. Bei Patienten mit Diabetes Typ II kommt es verstärkt zu Komplikationen, was eine erhöhte Morbidität und Mortalität zur Folge hat.

Ein hoher Insulinspiegel kann zur DNA-Oxidation und damit zu DNA-Schäden führen. Diverse Studien postulieren, dass Hyperinsulinämie ein entscheidender Risikofaktor für verschiedene Krebserkrankungen darstellt. Zur Untersuchung des Insulin-Signaltransduktionsweg, über den oxidativer Stress und daraus resultierender Genomschäden induziert werden, wurden aus diversen Pharmazeutika und Naturstoffen, welche den Insulin-Signalweg beeinträchtigen, PI3K Inhibitoren, Resveratrol, Lovastatin und RAD-001 aufgrund ihrer positiven Effekte bei Stoffwechselerkrankungen, ausgewählt.

Mit diesen Verbindungen wurde die anti-Genotoxizität (Schutzwirkung) hinsichtlich des durch Insulin induzierten oxidativen Stresses und Genomschadens in einer primären Nierenzelllinie der Ratte in vitro untersucht.

Unsere Ergebnisse zeigten protektive Effekte der ausgewählten Substanzen hinsichtlich genotoxischer Schäden sowie einen signifikanten Rückgang reaktiver Sauerstoffspezies bei insulinbehandelten Zellen, wobei der Wirkmechanismus zwischen den Substanzen jedoch unterschiedlich war. Somit handelt es sich bei den untersuchten Stoffen um äußerst interessante Verbindungen, die in der Zukunft Diabetes mellitus Therapien unterstützen könnten.

Außerdem untersuchten wir den Zusammenhang zwischen Schwangerschaftsdiabetes und Genomschäden in humanen Blutzellen. Dafür verwendeten wir neben humanen HL-60 Zellen nicht stimulierte sowie mit Hilfe von Phytohemagglutinin (PHA) zur Aufnahme des Zellzyklus stimulierte periphere mononukleäre Blutzellen von gesunden sowie von Gestationsdiabetes betroffenen Probandinnen. Wir analysierten zunächst den Einfluss von Östradiol, Progesteron, Adrenalin und Triiodthyronin auf den Genomschaden dieser Zellen in vitro.. Parallel dazu bestimmten wir in die basalen Genomschäden im Vollblut, in nicht stimulierten sowie in PHA-stimulierten peripheren mononukleären Blutzellen. Diese Studie schloss nicht-schwangere Frauen mit bzw. ohne Einnahme hormoneller Kontrazeptiva sowie je eine Subgruppen mit übergewichtigen Frauen, gesunden schwangeren Frauen und Frauen mit

Schwangerschaftsdiabetes ein. Bei Schwangeren wurde einige Zeit nach der Entbindung eine zweite Blutuntersuchung durchgeführt. Wir konnten zeigen, dass Frauen mit Schwangerschaftsdiabetes sowie übergewichtige Frauen im Vergleich zu normalgewichtigen, nicht-schwangeren Frauen sowie gesunden schwangeren Frauen mehr basale DNA-Schäden sowohl im Comet-Assay als auch im Mikrokern-Test in stimulierten peripheren mononuklearen Zellen aufweisen. Des Weiteren sanken die DNA-Schäden bei Frauen mit Schwangerschaftsdiabetes zwei Monate nach der Geburt. Darüber hinaus verstärkten die verwendeten Hormone in vitro die zellulären Genomschäden, jedoch überstiegen sie nicht die größere Menge an DNA-Schäden, welche bei übergewichtigen Frauen bzw. Frauen mit Schwangerschaftsdiabetes nachgewiesenen wurden.

Zusammenfassend lässt sich feststellen, dass Insulin Zellschäden in vitro induzieren kann, die jedoch durch Pharmazeutika und Naturstoffen reguliert werden können. Diese Erkenntnis könnte zukünftig Diabetespatienten helfen, die an Hyperinsulinämie leiden. Schwangerschaftsdiabetes, eine besondere Form des Diabetes, führt zu erhöhten DNA-Schäden bei betroffenen Frauen, die sich nach der Geburt jedoch wieder verringern. Die Zellen betroffener Frauen zeigen keine erhöhte, sondern vielmehr eine verminderte Sensitivität für weiteren DNA-Schäden durch hormonelle Behandlung in vitro. Eine mögliche Erklärung dafür könnte sein, dass eine maximal induzierbare Zahl an DNA-Schäden, die durch hormonelle Einflüsse bzw. daraus resultierenden Aktivierungen von Signalkaskaden hervorgerufen werden können, existiert.

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Affidavit

I hereby declare that my thesis entitled "**Modulation of insulin-induced genotoxicity in vitro and genomic damage in gestational diabetes**" is the result of my own work. I did not receive any help or support from commercial consultants or others. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I verify that this thesis has not been submitted as part of another examination process, neither in identical nor in similar form.

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Eman Da'as Awad