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Chapter 9

Relation of the Types of DNA Damage to Replication Stress and the Induction of Premature Chromosome Condensation

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Additional information is available at the end of the chapter

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1. Introduction

Any integrated view of the diversity of biochemical reactions involved in the faithful replication of eukaryotic chromosomes and their accurate mitotic segregation is not possible without careful consideration of the molecular mechanisms that are responsible for repairing damaged DNA. In order to arrange and order the sequence of events, in which the various levels of organization are only stages of the same molecular pathway, there is a need for both a timely switching on of numerous genes and the precise cooperation of large numbers of proteins. An important clue concerning the nature of the competitive interaction between these different elements comes from looking at the response to DNA damage.

The present chapter is a review of the types of DNA damage generated under stressful conditions and experimental approaches to the relation of these types of DNA damage to hydroxyurea treatment and caffeine-induced premature chromosome condensation (PCC). In this chapter, an attempt is also made to explain the molecular base of DNA damage and to present experimental procedures allowing the illustration of DNA damages at the cell level, especially with the use of histochemical and immunocytochemical methods. It will be experimentally shown, among others, that replication stress mainly leads to the generation of double-strand breaks in DNA (DSBs), while the breakage of restrictive interactions of checkpoints during PCC induction results in the accumulation of single-strand breaks (SSBs).

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2. The types and molecular base of DNA damage

DNA can be damaged by the action of endogenous (intrinsic) or exogenous (extrinsic) stress factors. The endogenous factors include, among others, errors generated during replication and reactive oxygen species (ROS). The exogenous (environmental) factors are divided into (i) physical factors, e.g. UV and ionizing radiation (X, γ); (ii) chemical factors, i.e. mutagenic polycyclic aromatic hydrocarbons (PAH), nitrosamines, dioxins, analogues of bases and alkylating agents; and (iii) biological factors, such as viruses.

Stress-induced damage includes spontaneous depurination and deamination, oxidation, formation of DNA adducts induced by alkylating agents, formation of cyclobutane dimers, single- and double-strand damage, as well as errors made during replication, repair, reverse transcription and recombination. DNA is also subject to covalent modifications that may affect nitrogen bases and lead to changes in base pairing between DNA strands, or even entirely preventing base pairing. Genomic instability may also be associated with chromosomal rearrangements which result from changes that occur in the *trans* position (including replication, DNA repair and S phase checkpoint pathways) or from changes that act in the *cis* position, i.e. in the regions of chromosomal instability, known as hotspots, for example breaks or fragile sites and highly transcribed DNA sequences (Aguilera & Gómez-González, 2008).

Plants, due to their 'settled' lifestyles are exposed to many environmental factors that cause disturbances in the cell cycle. They are often threatened by excessive salinity, drought, extreme low or high temperatures, as well as fungal or bacterial infections (Vashisht & Tuteja, 2006). Each of these burdens leads to the mobilization of defense responses: (1) activation of cell cycle checkpoints and DNA repair factors, (2) inhibition of cell growth, or (3) initiation of the apoptosis pathway (Deckert et al., 2009 and references therein).

Recognition of double-stranded breaks depends on the MRN complex (Mre11-Rad50-Nbs1), necessary for binding chromatin-remodeling factors (Schiller et al., 2012). MRN complex acts as a stabilizing platform for broken endings of DNA molecules. It binds to the sites of damage and ATM kinase, and promotes phosphorylation of histone H2A (H2AX-Ser139) and the processing of DNA. Processing of ends can either rely on their alignment, necessary to continue the connection through the induction of non-homologous end joining, or long single-stranded fragments for homologous recombination. Eukaryotic organisms use many types of DNA repair: (i) 3'-5' exonuclease activity of DNA polymerase; (ii) reversion repair (RR); (iii) mismatch repair (MMR); (iv) base excision repair (BER); (v) nucleotide excision repair (NER), (vi) non-homologous end joining (NHEJ); (vii) homologous recombination (HR); (viii) translesion synthesis (TLS). The methods also include: photoreactivation; methylguanine methyltransferase (MGMT), catalyzing the reaction of demethylation of methylated guanine bases; double strand break repair (DSBR); synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR).

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3. Replication stress and activation of checkpoint signaling pathways

Under the conditions of replication stress, the rate of DNA synthesis is slowed down and the possibility of entry into mitosis is blocked until the expression of specific genes and activation of repair factors. The control over DNA synthesis then involves a system of intra-S phase checkpoint, activated after the detection of DNA damage - in particular double strand breaks (DSBs) or single-strand breaks (SSBs) [Figure 1; (Bartek et al., 2004; Osborn et al., 2002; comp. Rybaczek & Kowalewicz-Kulbat, 2011)].

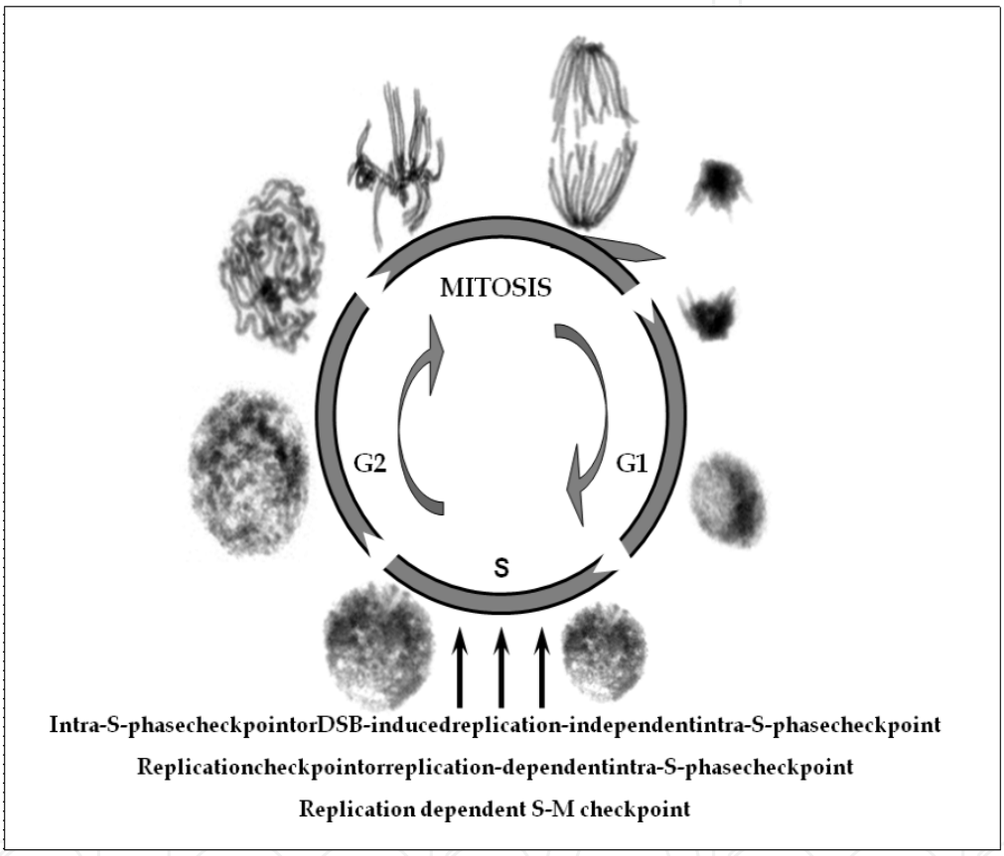


Figure 1. The three major S-phase checkpoints within the cell cycle

Further stages of the cell cycle are blocked until the repair of detected damage (Adamsen et al., 2011; Herrick & Bensimon, 2008). It has also been shown that any disruption of structural nature (e.g. DSB or SSB) induces a slowdown in the replication fork movement and further DNA damage, e.g. through the influence of replication inhibitors, may result in total inhibition of the cycle in the intra-S phase checkpoint (Blow & Hodgson, 2002; Elledge, 1996). Then checkpoint sensory factors trigger a signal transduction cascade, delivering a signal of DNA damage to effector proteins via transmitters (Mordes & Cortez, 2008; Nojima, 2006).

Thus, the detection of DSBs activates an ATM-dependent pathway (*Ataxia Telangiectasia Mutated*) and a slightly more slowly activated parallel ATR-dependent pathway (*Ataxia Telan-*

giectasia mutated – Rad3-related). The target substrate for both these sensory kinases is Cdc25 phosphatase (Cortez, 2003). The function of ATR kinase is not limited solely to the transmission of signals in response to DNA double breaks in the S phase checkpoint. This enzyme is activated during each S phase and plays an active role in regulating the initiation of DNA replication under physiological conditions. In addition, it is involved in the recognition of single-stranded DNA molecules (ssDNA; Shechter et al., 2004). ATR occurs in a durable complex with ATR-interacting protein (ATRIP), focusing in the area of the nucleus in regions corresponding to the sites of DNA damage (Myers et al., 2007). Research carried out on cytoplasmic extracts of *Xenopus* oocytes revealed that ATR associates with chromatin during DNA replication, and dissociates after its completion (Freire et al., 2006; Harper & Elledge, 2007; reviewed by Marheineke & Hyrien, 2004). The association of ATR and DNA breaks is also a result of the elimination of the replication factor A (RPA), while its appearance is independent of the presence of α-type DNA polymerase. Therefore it seems that the "recruitment" of ATR occurs after a partial generation of replication forks in the *origin* region, but before Polα association (Luciani et al., 2004; Namiki & Zaou, 2006; Zou & Elledge, 2003). Although ATR-ATRIP complexes can bind to certain DNA structures, their participation in the activation of cell responses to replication stress is not possible without the participation of two other factors: replication factor C (RFC) and proliferating-cell-nuclear-antigen-like proteins (PCNA-like). During replication, RFC recognizes the binding sites between primers/starters of RNA and DNA matrix and assembles PCNA, a toroidal homotrimer protein encircling DNA – also known as a "sliding clamp" which determines the processivity of the related DNA polymerases (Majka & Burgers, 2004; Tan et al., 2012). In the cells of *S. pombe*, Rad17 (RFC1 factor and four small subunits RFC2-5) and Rad9/Hus1/Rad1 (PCNA-like 9-1-1 complex), participate not only in the functional organization of the intra-S phase checkpoint, but also other cell cycle checkpoints whose function is to monitor the structural DNA damage [e.g. G2 (Majka et al., 2006, reviewed by Lin & Dutta, 2007)]. Recruitment of PCNA-like complexes to the sites of DNA damage in a molecule is, perhaps, independent of the activation of ATR and Chk1 (Niimi et al., 2008; Scorah et al., 2008), but is an important element of the mechanism signaling the appearance of structural disorders. In the cells of *S. pombe* and in mammals, Rad17 and Hus1 are factors determining the possibility of phosphorylation of Chk1 kinase by ATR. Rad17 is also a substrate of ATR. Although both these proteins bind to chromatin in intact cells, phosphorylation of Rad17 by ATR significantly increases with the increasing volume of PCNA-like complexes, following the occurrence of DNA conformational disorders. It therefore appears that the first stage of the then triggered signaling pathway is the independent localization of Rad17 and ATR-ATRIP complexes in the regions of damage; the next stage is a Rad17-dependent assembly of PCNA-like complexes around the DNA. PCNA-like complexes enable the activation of ATR molecules and - consequently - the phosphorylation of ATR substrates located within chromatin, such as Rad17 and Rad9 (Majka et al., 2006; Niida & Nakanishi, 2006). In addition to ATM and ATR kinases in humans, and their homologues in yeast cells, the PIKK family of signaling proteins includes also DNA-dependent protein kinase (DNA-PK). This enzyme consists of a DNA-PK catalytic subunit (DNA-PKCS,) and a heterodimeric subunit Ku70-Ku80. DNA-PKCS is a DNA-dependent serine-threonine kinase, showing a relatively weak ability to

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bind to DNA free ends; however, this affinity is enhanced and stabilizes under the influence of heterodimer Ku70-Ku80. It is believed that DNA-PK participates primarily in the repair of double-strand breaks (DSBs) by non-homologous end-joining [NHEJ (Müller et al., 2007; Pawelczak & Turchi, 2008; Shimura et al., 2007)].

Replication protein A (RPA) binds to all single-strand DNAs in the nucleus, including the parts of ssDNA formed during DNA replication and repair (Costanzo et al., 2003). The association of RPA and ssDNA (RPA-ssDNA) is an important component of signaling and the place to which the ATR molecule binds (this mechanism occurs both in human cells and in *S. cerevisiae*; Zou & Elledge, 2003). However, recognition of RPA-ssDNA structures and recruitment of other proteins to these complexes occur through the activity of ATRIP which occurs in conjunction with the ATR kinase. Biochemical studies indicate that ATRIP binds to the N-terminal part of the large subunit of RPA via its conserved acidic alpha-helix domain (Ball et al., 2007). The RPA-ssDNA complex is not a sufficient stimulus for binding the ATR-ATRIP complex and does not activate ATR. The induction and transmission of the signal "down" depends on ATR-ATRIP interaction with another protein complex, i.e. 9-1-1, which recognizes the DNA end adjacent to the RPA-coated ssDNA. The 9-1-1 complex is also responsible for recruiting TopBP1 protein, the main activator of ATR-ATRIP complex in the cells of vertebrates (Kumagai et al., 2006). In addition, the RPA-ssDNA platform recruits RAD17 and claspin, proteins strongly interacting with ATR, leading to the phosphorylation of ATR substrates, including Chk1 kinase (Bartek et al., 2004). Thus the presence of RPA is crucial for the specific recruitment of signaling factors to the 5' end of the damaged DNA (Ellison & Stillman, 2003). In this case, it is single-strand DNA fragments that are responsible for the activation of the checkpoint. Structures of this type are generated as a result of impaired DNA polymerase activity during replication, during the formation of double strand DNA breaks, at the ends of telomeres, and even during DNA repair via nucleotide excision. All of these factors activate the ATR kinase to recruit repair proteins (Byun et al., 2005; Cimprich & Cortez, 2008; Nedelcheva et al., 2005). Recent studies have shown that for the effective recruitment and signaling in response to DNA damage, ATR kinase requires continuous cooperation with its sister sensory ATM kinase, showing some similarity in structure and function (Cimprich & Cortez, 2008). These kinases also share phosphorylation substrates, e.g. H2AX histones (Burma et al., 2001; Ward & Chen, 2001).

4. Premature chromosome condensation and overriding of cell cycle checkpoint

The initiation of mitotic chromosome condensation in normal cells is preceded by the completion of all processes related to DNA replication and repair of abnormal DNA structures generated during the S phase. The main task of the checkpoint in G2 phase is to block cell entry into mitosis in the event of an anomaly in the genetic material. The common elements of the biochemical pathway that control the G2/M transition and of the S-phase checkpoint, are ATM and ATR kinases, and their role is to maintain the MPF complex, i.e. M-phase promoting factor (CDK1 kinase with cyclin B) in an inactive state

(Raleigh & Connell, 2000). Both in animal cells and in yeast, the activation of the CDK2-cyclin B complex, induced by phosphatase Cdc25, is a necessary condition for the initiation of mitotic chromosome condensation. The activation of ATM and ATR kinases during the G2 phase causes a cascade of phosphorylation. Similar to DNA replication, the substrates of these sensory kinases are the kinases Chk2 (for ATM) and Chk1 (for ATR). Chk1 kinase (active form) phosphorylates Cdc25 phosphatase by blocking its enzymatic activity (Cdc25 is then not able to carry out the activating dephosphorylation of CDK1 kinase; De Veylder et al., 2003). Phosphorylation of the phosphatase Cdc25 can lead to its degradation through ubiquitin-dependent proteolysis, or to association with 14-3-3 protein and consequently to its removal from the nucleus (Boutros et al., 2006). At the same time, ATM and ATR kinases induce gene expression of Wee1 kinase (responsible for blocking cell cycle progression in G2 phase), thus gaining the time required to repair defective DNA structures. Probably, the activation of Wee1 kinase also involves the activity of kinases Chk1 and Chk2 (De Schutter et al., 2007). In animal cells, ATM kinase also activates the p53 pathway. This factor is involved, among others, in the regulation of responses to replication stress, altered DNA structure, oxidative stress and osmotic shock, and disturbances in the integrity of cell membranes. Because of its multiple functions in cell cycle regulation, p53 has been termed 'the guardian of the genome' (Han et al., 2008).

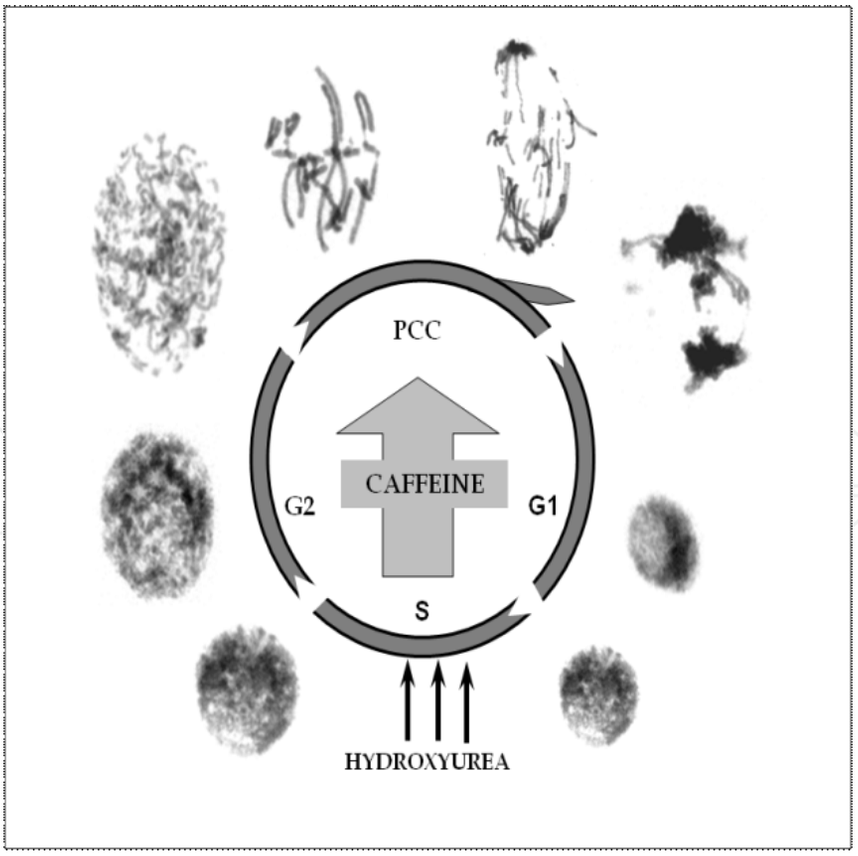


Figure 2. Overview of the induction of premature chromosome condensation (PCC)

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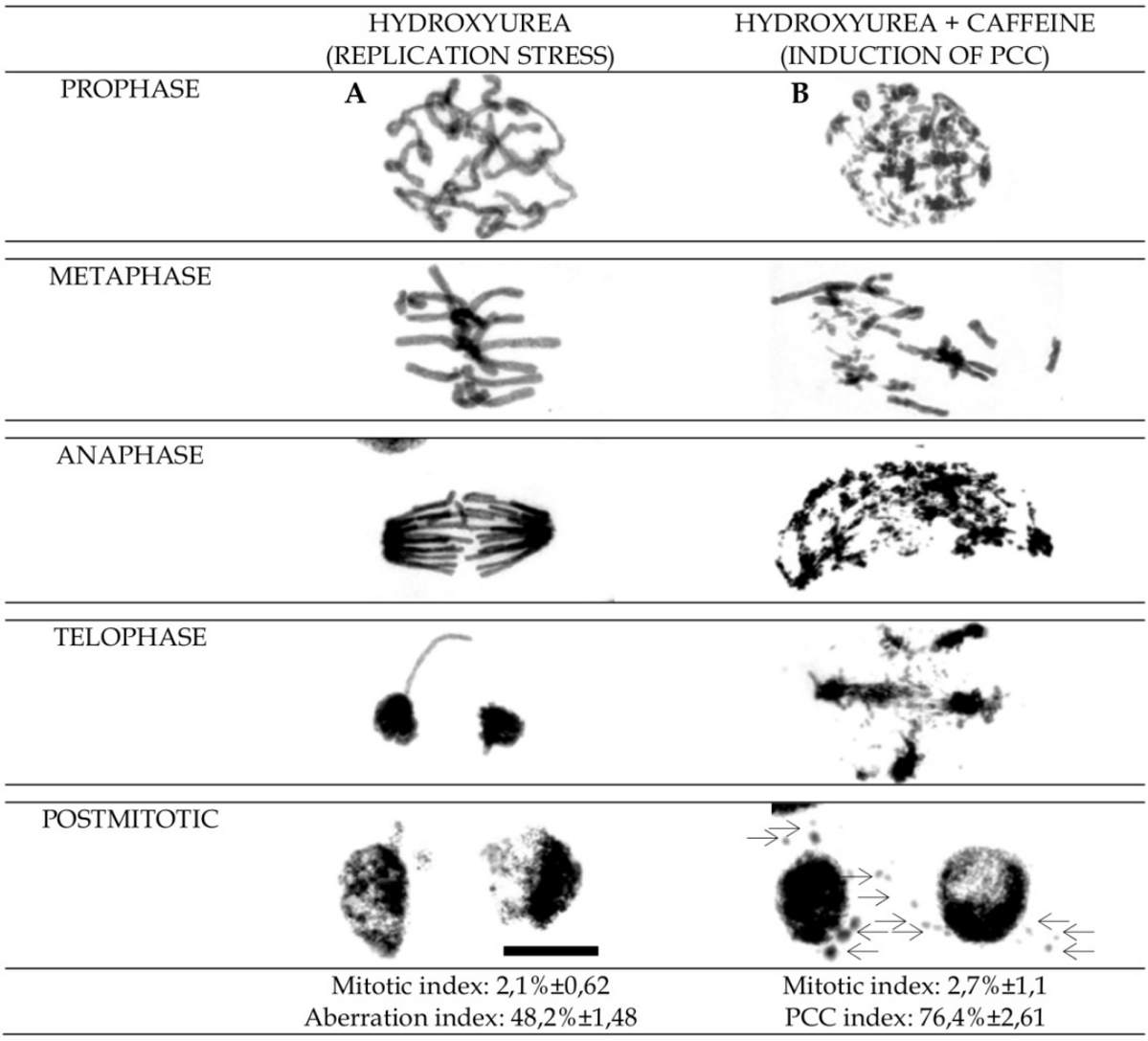


Figure 3. Feulgen-stained root meristem cells of *Vicia faba*: (A) hydroxyurea-treated (2.5 mM, 24 h); (B) caffeine-induced PCC (2.5 mM HU for 24 h → the mixture of 2.5 mM HU and 5 mM CF for 8 h). The array of aberrations in serie 'A' included a relatively small number of breakpoints per cell nucleus (≤ 5). The full array of aberrations (≥ 25 per cell nucleus) in serie 'B' included chromosomal breaks, irregular condensation/decondensation of chromatin, lost and lagging chromatids and chromosomes as well as segregation defects. Micronucleus formation (arrows), were found significantly increased in comparison either with the control or HU treatment (comp. Rybaczek & Kowalewicz-Kulbat, 2011; Rybaczek et al., 2008). The mitotic index was calculated as the percent ratio between the number of dividing cells and the entire meristematic cell population. Index of aberrations was calculated as the percent ratio between the number of cells showing chromosome aberrations and all mitotic cells. PCC index was calculated as the percent ratio between the number of cells showing chromosome aberrations typical of premature mitosis and all mitotic cells. Experimental procedure of Feulgen staining: root tips were fixed in cold absolute ethanol and glacial acetic acid (3:1, v/v) for 1 h, washed several times with ethanol, rehydrated, hydrolysed in 4 M HCl (1.5 h), and stained with Schiff's reagent (pararosaniline; Sigma-Aldrich) according to standard methods. After rinsing in SO_2-water (3 times) and distilled water, 1.5 mm long apical segments were cut off, placed in a drop of 45% acetic acid, and squashed onto microscope slides. Following freezing with dry ice, coverslips were removed and the dehydrated dry slides were embedded in Canada Baume. Slides were analysed under the light microscope to count mitotic cells that had characteristic features of either normal mitosis or PCC. *Bar* 20 µm

In a cell there are also mechanisms responsible for DNA damage tolerance (DDT), which allow the completion of the replication of genetic material despite the damage to DNA that blocks replicase complex. In addition, disruption of the efficiency of the intra-S phase checkpoint, following the action of chemical agents, leads to the induction of premature chromosome condensation (PCC; Figure 2), specifically via overriding of the control over the stability of the genome, even despite the uncompleted S phase and not implemented post-replication repair processes in G2 phase (Figure 3A). The successive phases of prematurely initiated mitosis follow an aberration course because the unreplicated regions of the genome are manifested in the form of losses or breaks in chromosomes [(Figure 3B) comp. Rybaczek et al., 2008; Rybaczek, 2011]. Caffeine (CF) is a particularly effective PCC inducer. It blocks the activity of kinases ATM/ATR (Cortez, 2003), by which they can not phosphorylate their downstream kinases (i.e. Chk1 and Chk2; Rybaczek & Kowalewicz-Kulbat, 2011; Rybaczek et al., 2007) and, consequently, catalytic activity of Cdc25 phosphatases is maintained - phosphatases which serve as inducers of complexes CDK1-cyclin B (MPF; M-phase Promoting Factor) and trigger mitotic phosphorylations (Gotoh & Durante, 2006; Rybaczek & Kowalewicz-Kulbat, 2011).

The overriding of the checkpoint function induced by the action of caffeine leads to the selective sensibilization of pro-oncogenic cells deprived of p53 protein and tumorous cells to the action of antineoplastic factors and the effect of ionizing radiation (Yao et al. 1996). The test results obtained by Wang and co-workers (1999) show that the effectiveness disturbance of the S-M control system induced by caffeine in *S. pombe* cells is connected with the activation of Cdc2 kinase (due to the removal of phosphate group from Tyr15 within the ATP-binding pocket) and with the septation process that during a normal course of cell cycle of *S. pombe* results from the transfer through mitosis.

5. Labeling of DNA damages following hydroxyurea-induced stress and caffeine-induced premature chromosome condensation

One of the basic protective mechanisms of the replicative apparatus are foci concentrating molecules of phosphorylated histones H2AX (Rybaczek & Maszewski, 2007a; Rybaczek & Maszewski, 2007b). The generation of γ-H2AX molecules as a result of exposure to stressors is a rapid process. Half of the γ-H2AX histones appear as early as after 1 min of irradiation and a maximum level is reached with 3 to 10 minutes of exposure; then, in terms of 1 Gy radiation, γ-phosphorylation concerns approximately 1% of histone H2AX molecules, which is equivalent to about $2x10^6$ base pairs of DNA in the region of the double-strand break (DSB). It is assumed that each grouping of these molecules determines a single DSB region (Paull et al., 2000; Rogakou et al., 1998). Phosphorylated histone H2AX binds cohesin and chromatin-modifying complex NuA4. The acetylation of histones follows, which allows connection of the INO80 complex, which removes histones in the area of the damaged DNA, thereby creating single-strand regions. This greatly simplifies the recruitment of proteins of the pathway of response to DNA damage and repair proteins. Then TIP60 complex is connected, followed by the removal of dimers H2AX/H2B and insertion of non-phosphorylated

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histone H2A, and thus switching off the signal of the DNA structure checkpoint and - after the completion of repair - restoration of the correct chromatin structure. The results of testing using antibodies recognizing phosphorylated histone H2AX (α-H2AX^{S139}) - microscopic images of immunofluorescence in meristematic root cells of *Allium porum*, *Vicia faba*, *Raphanus sativum*, and HeLa cells, and strong signals obtained using a Western blot – provide, above all, the next example of homology of organization of cellular systems in animals and plants - the similarities in their structural elements, systems, and hence, similarities of biochemical regulatory mechanisms (Rybaczek & Kowalewicz-Kulbat, 2011; Rybaczek & Maszewski, 2007a). Our studies have shown that a significant level of Ser139 phosphorylation in histone H2AX appears after hydroxyurea treatment, as it was the case with phosphorylations of Chk1 serines 317 and 345. Correlation of immunolabeling using anti-Chk1 (Ser317) and anti-H2AX (Ser139) antibodies, especially evident at the boundaries of nucleolar and perinucleolar regions of chromatin, seems to indicate that both regions overlap with the areas of an increased activity of Chk1 kinase (Rybaczek & Maszewski, 2007b). It was also concluded that as opposed to *V. faba* and *A.porrum* (both representing a 'reticulate' type of DNA package) the diffuse chromatin in chromocentric cell nuclei of *R. sativus* may be more vulnerable both to generate DSBs and to recruit repair factors (Rybaczek & Maszewski, 2007a). The formation of histone H2AX foci phosphorylated at Ser139 is therefore a sensitive test showing the presence of structural damage to the genome (Figure 4A, B). An equally sensitive test detecting single-strand DNA damage is labeling nuclei by antibodies recognizing single-stranded DNA (anti-single-stranded DNA, Figure 4A, B) or antibodies recognizing *PARP2* gene product, i.e. Poly(ADP-Ribose) Polymerase-2 (PARP-2;Figure 5A, B).

Comparisons of means were made using nonparametric Mann-Whitney U tests, due to the fact that some series had a skewed distribution (Figure 4A). The following has been indicated: (i) a significant increase in the DSB series compared to SSB in the control series ($U = 6.23$; $P \leq 0.001$), (ii) a significant increase in the DSB series compared to SSB after a 24-hour activity of 2.5 mM hydroxyurea ($U = 8.61$; $P \leq 0.001$), and (iii) a significant increase in SSB compared to DSB in the series in which PCC induction was performed under the influence of 5 mM caffeine (under constant sustained hydroxyurea stress; $U = 8.61$; $P \leq 0.001$).

Additionally, the presence of double-stranded breaks (DSBs) in the nuclei of cells undergoing PCC suggests also that premature entry into mitosis occurs before the completion of DNA repair (Rybaczek et al. 2007; Rybaczek et al. 2008). The key target of S-M checkpoint is the activity of the cyclin B/Cdk1 complexes (MPF), but similar effects can result from the change in the activity balance of protein kinases and phosphatases brought about, e.g. by the hyperexpression of *cdc25* genes (Forbes et al. 1998).

PARP activation is an immediate cellular response to chemical or radiation-induced DNA SSB damage. PARP-2 is a nuclear protein whose main role is to detect and signal SSB to the enzymatic machinery involved in the SSB repair. Once PARP detects a SSB, it binds to the DNA, and, after a structural change, begins the synthesis of a Poly(ADP-Ribose) chain (PAR) as a signal for other DNA-repairing enzymes such as DNA ligase III (LigIII), DNA polymerase beta (polβ), and scaffolding proteins such as X-ray cross-complementing gene 1 (XRCC1). After repairing, the PAR chains are degraded via PAR glycohydrolase [(PARG) Isabelle et al., 2010].

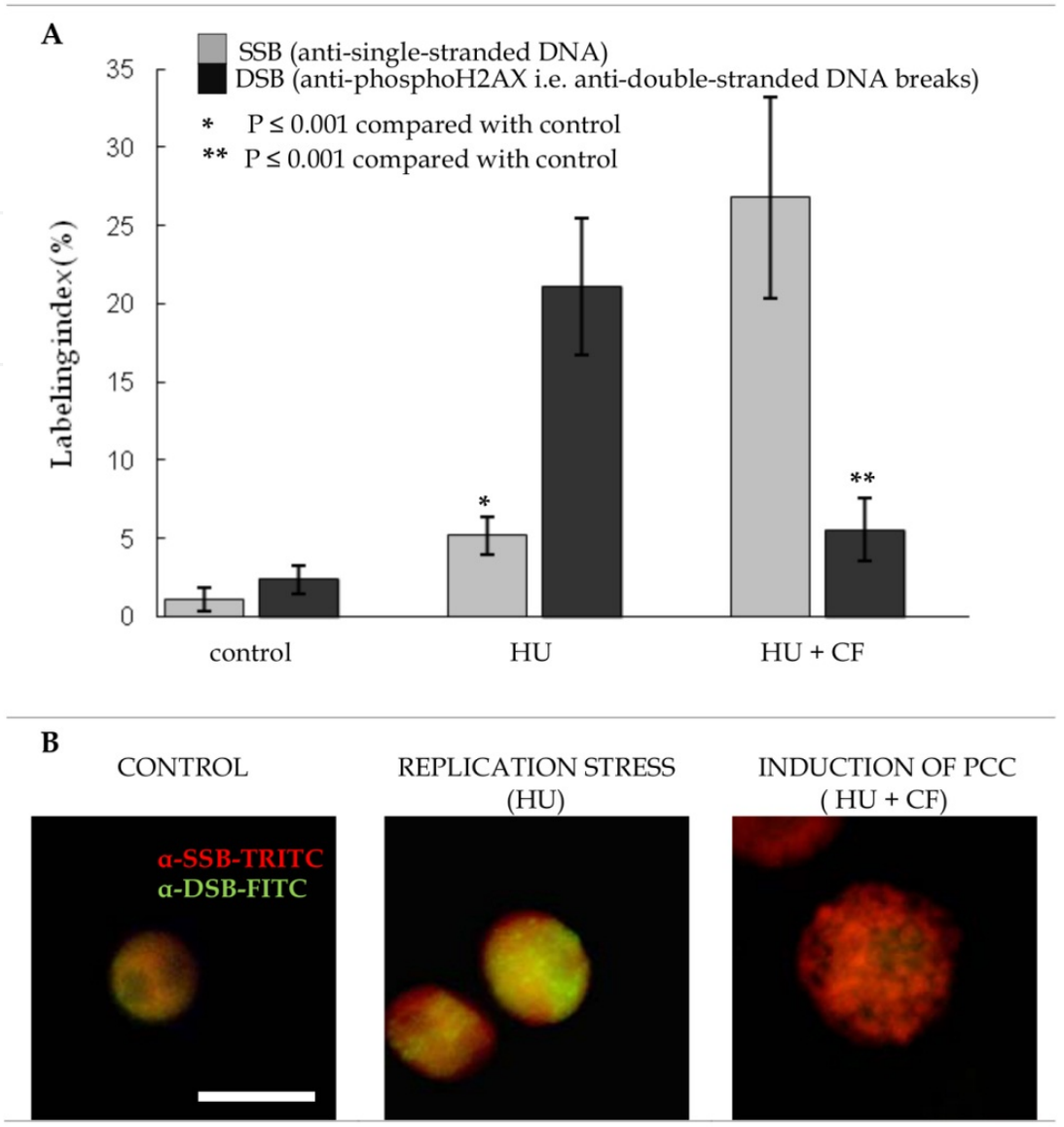


Figure 4. Immunolabeling indices (%) estimated for *Vicia faba* stained with anti-ssDNA [red, TRITC-labeled] and anti-H2AX(Ser139) [green, FITC-labeled] antibodies. *Columns*, mean from five independent experiments; *bars*, SD. For immunocytochemical detection of single-standed DNA and phospho-H2AX histone cells were fixed for 45 min in 4% formaldehyde buffered with PBS. Excised apical parts of roots were then placed in a citric acid-buffered digestion solution (pH 5.0; 37°C for 45 min) containing 2.5% pectinase (Fluka), 2.5% cellulase (Onozuka R-10; Serva) and 2.5% pectoliase (ICN). The cells were pre-treated in a blocking buffer (10% horse serum, 1% bovine serum albumin; BSA, 0.02% NaN_3, 1 x PBS) for 1 h at room temperature to minimize the non-specific adsorption of the antibodies to the coverslip, and were incubated overnight in a humidified atmosphere (4°C) with primary antibody. Mouse monoclonal antibody to single-stranded DNA was used at 1:200 (MILLIPORE), rabbit polyclonal antibody to phospho-H2AX (Ser139) was used at 1:750 (CELL SIGNALING). Secondary antibodies, including FITC-conjugated goat anti-rabbit (for H2AX), and TRITC-conjugated goat anti-mouse antibodies (for ssDNA), were used at 1:1000 for 1 h at room temperature in the dark. Secondary antibodies were from Sigma-Aldrich. The labeling index was calculated as the ratio of immunofluorescence-labeled cells to all cells in a meristematic population. *Bar* 20 μm

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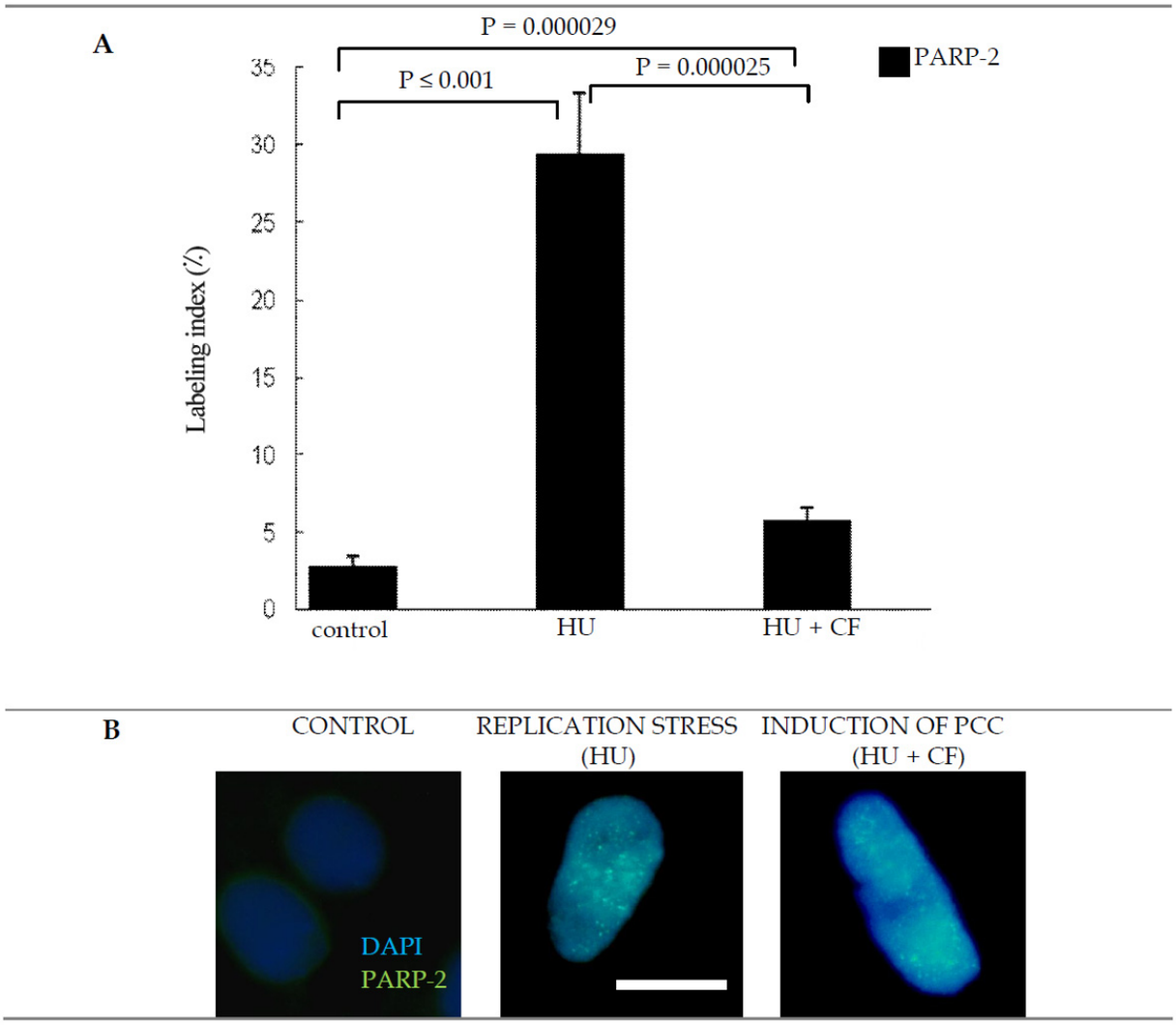


Figure 5. Fig. 5. Immunolabeling indices (%) estimated for *Vicia faba* stained with anti-PARP-2 antibody [green, Dy-Light®488] and DAPI [blue]. *Columns*, mean from five independent experiments; *bars*, SD. For immunocytochemical PARP-2 (Poly[ADP-Ribose] Polymerase-2) cells were fixed for 45 min in 4% formaldehyde buffered with PBS. Excised apical parts of roots were then placed in a citric acid-buffered digestion solution (pH 5.0; 37°C for 45 min) containing 2.5% pectinase (Fluka), 2.5% cellulase (Onozuka R-10; Serva) and 2.5% pectoliase (ICN). The cells were pre-treated in a blocking buffer (10% horse serum, 1% bovine serum albumin; BSA, 0.02% NaN_3, 1 x PBS) for 1 h at room temperature to minimize the non-specific adsorption of the antibodies to the coverslip, and were incubated overnight in a humidified atmosphere (4°C) with primary antibody. Rabbit polyclonal antibodies specific to PARP-2 were purchased from AGRISERA (at a dilution of 1:50). Bound primary antibodies were detected with secondary goat anti-rabbit IgG Dy-Light®488 antibody (AGRISERA; at a dilution of 1:1000, for 1 h at 18°C). Nuclear DNA was stained with 4',6-diamidino-2-phenyl-indole (DAPI, 0.4 µg/ml; Sigma-Aldrich). The labeling index was calculated as the ratio of immunofluorescence-labeled cells to all cells in a meristematic population. *Bar* 20 µm

Nonparametric Kruskal-Wallis tests were used for analysis of variance (H = 78.9; P ≤ 0.001; Figure 5A). Comparisons between groups were made using post hoc tests (Figure 5A). A

statistically significant increase in the fluorescence labeling index of the anti-PARP2 in series HU and PCC was observed relative to the control, as well as a significantly higher labeling index for HU compared to the PCC series (Figure 5A).

In summary, this chapter aims to review how the nature of the damage to nucleobases influences DNA repair with regards to DSB and SSB generation (Figures 4, 5). Reports, literature and our own research results show histone H2AX phosphorylated at Ser139 is the marker of double-strand breaks (Figure 4A, C). It was shown that rapid and sensitive detection of single-strand damage is possible thanks to immunocytochemical reaction performed using commercially available antibodies recognizing ssDNA (anti-ssDNA, MILLIPORE, Figure 4B, C), or another similarly useful SSBs marker, Poly(ADP-Ribose) Polymerase-2 (AGRISERA, Figure 5A, B). We demonstrate that replication stress leads mainly to the generation of double-strand breaks in DNA (DSBs), while the breakage of restrictive interactions of checkpoints during PCC induction results in the accumulation of single-strand breaks (SSBs).

6. Future perspectives and the key questions that remain unanswered

The formation of DNA damage is a continuous process. Out of necessity, it must be perceived in terms of temporal and spatial chromatin dynamics, and as coupled with the activation of checkpoints (Zhou & Elledge, 2000; Liu et al., 2006). The consequence of this activation is possibly the most efficient (i.e. fast and effective) initiation of the repair processes. Maintaining the efficiency is important, as any decrease in DNA repair efficiency, for example resulting from mutations in genes encoding repair proteins, may lead to neoplasia.

Most recent studies on DNA repair have been aimed at achieving various strategic objectives, most often concerned with strengthening the effects of widely understood radio and chemotherapy (Legerski, 2010). Thoms and Bristow (2010) describe the achievement of the "therapeutic ratio" as the primary aim of their investigations. Other researchers emphasize the benefits of mathematical methods in either future experimental studies of DNA repair or clinical studies of drug resistance (Lavi et al., 2012).

DNA repair processes have been studied using (i) different experimental systems, e.g. *in vitro* model (Garner & Costanzo, 2009), (ii) different cell types, e.g. human stem cells (Rocha et al., 2013) or even neurons (McMurray, 2005); (iii) model organisms, e.g. *Arabidopsis thaliana* cells, *Xenopus laevis* egg cell free extract (Garner & Costanzo, 2009); (iv) different proteins e.g. cyclin-dependent kinases (CDKs; Yata & Esashi, 2009), histone variants (Shi & Oberdoerffer, 2012) or cell cycle checkpoints connected proteins (Liu et al., 2006); as well as (v) the context of chromatin condensation (Shi & Oberdoerffer, 2012).

Most (although not all) molecular mechanisms involved in DNA repair appear to be evolutionarily conservative. However, many important questions still remain unanswered. This is particularly evident in studies on chromatin adopting different conformations and damaged - with varying intensity - by various factors and various states of condensation. This variety makes it difficult to draw definite conclusions with regard to the processes of DNA repair in chromatin fibres. In addition, the common features of almost all types of repair (concerning

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either SSBs or DSBs) is that they involve large protein complexes, and that the repaired DNA is subject to many structural changes not only initially but also during repair itself (e.g. unwinding or nucleolytic processing). Finally, control systems of higher plant cell cycles involve regulatory factors related to the "permanently embryonic" nature of meristematic zones, autotrophic metabolism, spatial stabilization, the presence of cellulose wall and the resulting specific intertissue dependencies (Jacobs, 1992). Hopefully, cutting-edge research techniques will soon make it possible to reveal many of the still unknown mechanisms of DNA repair and to formulate really definite conclusions.

7. Conclusion

The instability of the genome, visible in chromosome mutations and rearrangements, is usually associated with a pathological disorders, but is also of key importance for evolution. Processes that make up the cell cycle (replication, chromatin condensation, anaphase-telophase chromosome segregation and cytokinesis) occur in a sequential manner and are subject to precise control. However, the cell cycle includes several functionally different cycles that are inherently related to the cell cycle but independent of each other, for example, nuclear DNA cycle, nuclear membrane cycle, nucleolus cycle, microtubular cycle, a cycle of biosynthesis and segregation of cell organelles, and the use of sucrose like highly-energetic substances. Despite the enormous diversity of processes occurring in the cell cycle, the mechanisms responsible for the integrity of the genome exhibit a remarkable homology and coherence of action in reducing the effects of DNA damage. This results in the evolutionary development of organisms and an increase in their productivity in the expansion to new and more demanding environments.

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