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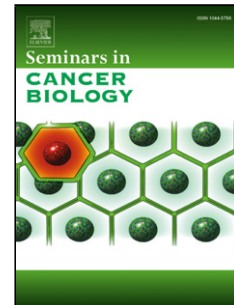
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## Accepted Manuscript

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**Effects of hyperthermia as a mitigation strategy in DNA damage-based cancer therapies**

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**Abstract**

Utilization of thermal therapy (hyperthermia) is defined as the application of exogenous heat induction and represents a concept that is far from new as it goes back to ancient times when heat was used for treating various diseases, including malignancies. Such therapeutic strategy has gained even more popularity (over the last few decades) since various studies have shed light into understanding hyperthermia's underlying molecular mechanism(s) of action. In general, hyperthermia is applied as complementary (adjuvant) means in therapeutic protocols combining chemotherapy and/or irradiation both of which can induce irreversible cellular DNA damage. Furthermore, according to a number of *in vitro*, *in vivo* and clinical studies, hyperthermia has been shown to enhance the beneficial effects of DNA targeting therapeutic strategies by interfering with DNA repair response cascades. Therefore, the continuously growing evidence supporting hyperthermia's beneficial role in cancer treatment can also encourage its application as a DNA repair mitigation strategy. In this review article, we aim to provide detailed information on how hyperthermia acts on DNA damage and repair pathways and thus potentially contributing to various adjuvant therapeutic protocols relevant to more efficient cancer treatment strategies.

**Keywords:** hyperthermia; DNA damage; DNA repair; cancer therapeutics; cancer treatment

## 1. Introduction

Hyperthermia is defined as the application of exogenous heat induction and has been well known for its therapeutic effects against several diseases including malignancies. Since the 1970s and 80s, several studies have focused on understanding the biology behind hyperthermia's beneficial effects on various cancer treatments. Heat induction is most commonly applied in combination with other primary therapies (e.g. radiation therapy and/or chemotherapy) since it can enhance their therapeutic effectiveness [1-3]. Results from *in vitro* and *in vivo* studies have shown that hyperthermia in the range of 41-47°C exerts various effects including i) direct killing of tumor cells, ii) alterations in the tumor microenvironment, iii) induction of heat shock proteins, iv) activation of the immune response, v) induction of the apoptotic cascade, vi) improvement of therapeutic outcome when applied with other treatments, vii) changes in cell cycle regulatory signaling pathways and viii) alterations in blood flow, oxygen and nutrients' distribution in the tumor site. From hot water pads to more technologically advanced hyperthermia platforms (utilizing different energy sources such as radiofrequencies, microwaves and laser), the main aim is to maintain the temperature elevation at the level of whole body (whole-body hyperthermia) and/or at the specific tumor site (local or regional hyperthermia) [4,5].

Earlier studies have demonstrated that apart from its cytotoxic properties, heat induction can also act as a “sensitizer” to other DNA damage-based treatment modalities. In fact, numerous studies have shown that hyperthermia in combination with radiation and/or various other chemotherapeutic drug treatments can induce significant antitumor effects under *in vitro* and *in vivo* experimental settings. Moreover, such studies have suggested that heat induction can cause DNA damage directly [6] and/or interfere with DNA repair pathways [7] thus enhancing the outcome of antineoplastic strategies. To these ends, hyperthermia not only affects a single DNA repair pathway but rather many different ones thus resulting in the

accumulation of damage lesions which in turn leads to cell death. Therefore, although enhancing our understanding of the underlying mechanisms by which hyperthermia can influence DNA damage is quite intriguing, it is also of great importance when looking into improving current and designing new, novel and innovative therapeutic approaches. Finally, this review article aims to describe the effects of hyperthermia as a mitigation strategy in DNA damage-based cancer therapies due to its ability to influence DNA damage and repair pathways and how such knowledge is employed in order to establish adjuvant cancer treatments of greater therapeutic effectiveness.

## **2. Hyperthermia and DNA damage response**

### **2.1 Direct Effects**

The prospect of hyperthermia inducing DNA damage directly rather than by other indirect means (i.e. ROS generation) has been the subject of several studies. Results from initial reports demonstrated that heat elevation can lead to the generation of DNA breaks after exposing Chinese hamster ovary cells to 45°C [6]. Additional studies using HeLa cells have showed that heat treatment at 43°C resulted in the induction of DNA lesions observed as early as a 15min of exposure. Moreover, it was evident that there was a strong correlation between the amount of hyperthermia-induced DNA strand breaks and cytotoxicity [8]. In an attempt to further characterize DNA breakage (in CHO cells following heat treatment), elevated levels of apurinic/apyrimidinic or abasic sites were observed [9]. In addition, work by other groups has suggested that although hyperthermia cannot induce the generation of double strand breaks (DSBs) by itself, it can alter the rates of observed DSBs (in radio-sensitization experiments) by increasing the levels of slowly rejoined DSBs instead [10]. Furthermore, experiments with primary human fibroblasts have also demonstrated that hyperthermia does affect the kinetics of DNA repair mechanisms by considerably slowing down cells. Elevated levels of mis-

rejoined and non-repairable DSBs were accompanied by a drop in the rates of successfully completed DNA repair after heat exposure in combination with irradiation. To this end, it was supported that hyperthermia by promoting delayed DNA repair processes can have an impact on the observed increase on levels of DNA breaks, after irradiation, which if not correctly repaired they will either stay non-rejoined or rejoin with a wrong broken end. It has been postulated that the latter hypothesis is more likely to occur due to their higher detection levels [11].

Evidence from several hyperthermia-induced radio-sensitization studies has shown that heat induction can affect DNA repair pathways as it restricts replication by reducing the activity of DNA polymerases, thus eventually increasing the appearance of more DNA breaks after irradiation [12-14]. In addition, data from early research reports suggested that heat treatment can promote topoisomerase inactivation in HeLa S3 cells [15] while other studies conducted in human epidermoid cancer (KB) cells have reported increased transcription and translation levels of topoisomerase II after exposure to 42°C or 45°C. To this end, treatment of KB cells with the etoposide VP-16 (a topoisomerase II targeting drug) was accompanied by further potentiation of cytotoxicity following these hyperthermia exposures [16]. The nature of these contradicting results (e.g. decreased enzyme activity or upregulation of expression levels of topoisomerase II) may be the end result of variations in experimental conditions regarding the duration and/or temperature of exposures in addition to the usage of different cell types. On the other hand, later research reports have outlined the induction of DSBs along with focal phosphorylation of histone H2AX (at Ser139) in response to heat treatments in H1229 cells. In general,  $\gamma$ H2AX foci are considered to be indicators of DSBs [17] whereas other studies have addressed the importance of H2AX's role in recruiting several repair factor molecules to areas exposed to DNA damage [18]. A number of studies investigating the induction of  $\gamma$ H2AX foci formation by hyperthermia have resulted in the publication of

opposing results. For instance, some of these studies support a direct association of  $\gamma$ H2AX foci with DSBs whereas other findings support an indirect role where H2AX phosphorylation occurs due to other cellular processes being “disturbed” by hyperthermia. The generation of  $\gamma$ H2AX foci was found to increase linearly in an exposure duration-dependent manner when exposing cells at temperatures from 41.5°C to 45.5°C. In addition, the contribution of each cell cycle phase to enhancing the formation of  $\gamma$ H2AX foci was studied with cells in the S phase exhibiting significantly more potential compared to those at the G1 or G2-M phase, thus suggesting the involvement of DSBs induction in triggering hyperthermia-induced cell death pathways [19]. Moreover, findings from subsequent studies confirmed that hyperthermia can promote the formation of  $\gamma$ H2AX foci, whilst also revealing the dependence of this process on ataxia-telangiectasia mutated protein (ATM). To this end, heat elevation promotes the auto-phosphorylation of ATM [at Ser(1981)] while also triggering its kinase activity. Experiments on mouse kidney fibroblasts with ( $Atm^{+/+}$ ) and without ( $Atm^{-/-}$ ) the ability to express ATM led to an elevation in the levels of  $\gamma$ H2AX foci in  $Atm^{+/+}$  cells and just a minimal increase in the case of  $Atm^{-/-}$  cells following thermal treatments. This observation, in turn, indicates that ATM's presence is necessary for H2AX phosphorylation and foci formation [20]. On the other hand, the same study argued that the heat-induced  $\gamma$ H2AX foci response does not relate to direct DNA damage due to the observed absence of foci formation of both DSBs repair factors namely 53BP1 and SMC1 [20]. Interestingly, the absence of DNA damage and chromosome aberrations could support the assumption that hyperthermia might cause chromatin alterations which are able to induce the cellular DNA damage response (DDR), i.e. activation of ATM, without the presence of apparent DNA damage [20]. Indeed, follow up studies have shown that DNA damage is not essential for the induction of DDR which can also be activated when single DNA repair factors remain physically attached on chromatin, thus indicating the importance of this interaction for triggering the DDR signaling



pathway [21]. Additional work by other groups further support the view that hyperthermia-induced  $\gamma$ H2AX foci does not depend on the generation of DSBs, as there was no evidence associating  $\gamma$ H2AX foci formation with cell death induction in various cell lines which were either resistant or sensitive to heat [22]. Finally, the same group outlined the role of heat induction in decelerating the formation of the  $\gamma$ H2AX/MDC1/53BP1 complex, which is one of the first events in the cellular response to formation of DSBs during heat radio-sensitization experiments [23].

Only recently, other reports have shown that cells in different cell cycle phases can be affected in a different manner by hyperthermia. To this end, it was reported that an increased number of DSBs (accompanied by H2AX phosphorylation) was observed when cells in G1 or G2 phase were subjected to hyperthermia compared to those in S phase [24]. More specifically, it was the generation of SSBs that was detected rather than that of DSBs supporting the hypothesis that heat elevation may interfere with the DNA replication process and thus slowing down its progression. Furthermore, it was also found that there was an appearance of  $\gamma$ H2AX foci at the sites of arrested replication forks which were suggested to prevent their collapse [24]. Obviously, there is conflicting evidence whether or not hyperthermia can trigger direct induction of DSBs thus highlighting the need for more sensitive detection methodologies in order to elucidate any underlying mechanism(s). Nevertheless, there is no proof indicating that heat induction can influence only a particular DNA repair pathway but it rather seems that it can affect multiple targets at different levels (Fig. 1).

## **2.2 Indirect Effects**

Although there are numerous studies supporting the ability of hyperthermia to directly induce the formation of DSBs thus affecting cellular DNA damage response, there are

also other indirect means leading to triggering this process (Fig. 2). For instance, one of the major causes of activating the DNA damage response is oxidative stress which is the result of an imbalance between production of reactive oxygen species (ROS; the by-products of oxygen metabolism in cell respiration) and the availability of effective antioxidant defenses. It is well documented that when the normal redox state of the cell is disturbed (due to excess increase of ROS) there is increased generation of peroxides and other free radical species within cells all of which will eventually lead to damaging major cellular macromolecules like protein, lipids and DNA [25]. To this end, several studies have associated hyperthermia with increased ROS production [26]. For instance, there is evidence demonstrating that hyperthermia can disturb the mitochondrial membrane, consequently leading to alterations in the cellular redox status [27,28] which in turn can result in DNA damage induction. Alternatively, ROS generation can cause extensive mitochondrial DNA damage which is a more persistent type compared to the nuclear one [29].

There are three well described checkpoints associated with the cell cycle: the G1, G2/M and the metaphase checkpoints [30]. In the context of DNA damage, there are two kinases acting as sensors according to the type of damage present, namely ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia Telangiectasia mutated and Rad3 related). It has been shown that ATM is activated by a complex consisting of the Mre11, Rad50 and Nbs1 proteins (MRN) in response to DSBs mostly in the G1 phase [31]. On the contrary, ATR is triggered by SSBs at the site of stalled replication forks in S phase while its activation also takes place as a consequence of the ATM induction by DSBs [32,33]. These two kinases phosphorylate and activate several other repair factors all of which result in the activation of a particular type of DNA repair mechanism based on a specific cell cycle phase of the cell [34]. The effects of heat elevation on mammalian cells appear to be dependent on the cell cycle phase of the cell with early studies indicating that cells in the S phase being more sensitive to

hyperthermia compared to those in the G1 and G2 phases [35]. Although, later reports have demonstrated that unsynchronized cells exposed to hyperthermia are often the subject of cell cycle arrest, this appears to be the case only with certain cell lines whereas others are not affected [36]. In addition, the duration and temperature of hyperthermia are equally important for determining the activation of cell cycle checkpoints, activation of DNA damage and/or heat shock proteins and induction of apoptosis [37].

Hyperthermia-induced activation of ATR leads to the phosphorylation of Chk1 both of which are involved in cell cycle arrest at the G2/M phase and consequently appear to be reducing the induction of cell death. Alternatively, hyperthermia can create chromosomal lesions which if not removed during mitosis can result in triggering cell death pathways [38]. As previously mentioned, hyperthermia has the ability to interfere with and/or stop the extension of replication forks [24]. Such replication retardation is followed by the re-localization of the proliferating cell nuclear antigen (PCNA; an important replication protein) to the nuclear matrix together with relocation of nucleolin to the nucleolus, with the later taken place 1-2h after exposure to hyperthermia when DNA replication remaining decelerated for up to 8h [39,40]. As discussed earlier, accumulation of SSBs at the S phase in response to heat exposures can be the outcome of the stalled replication forks and/or the effects of increased levels of ROS that, in turn, can cause DNA modifications and breaks. The appearance of SSBs can be correlated with the elevation of  $\gamma$ H2AX foci levels as this can occur at the DNA regions affected by replication or repair stress [41]. The latter may be the reason why several groups have not determined the induction of DSBs while detecting increased levels of phosphorylated H2AX [42,43].

In the occurrence of DSBs, there is a rapid increase in the generation of  $\gamma$ H2AX foci which is accompanied by the activation of ATM and thus playing a critical role in the phosphorylation of H2AX following exposure to hyperthermia. Higher rates of

phosphorylated H2AX have been observed in cells at the S phase when compared to those at the G1 and G2 phases [44]. DNA dependent kinase (DNA-PK) has the ability to phosphorylate H2AX at the replication forks in S phase cells after heat induction [24]. Moreover, other reports have proposed a protective role of DNA-PK and  $\gamma$ H2AX in genome integrity as they act by preventing formation of DSBs at replication sites [45]. The first step in heat induced activation of ATM is the formation of the MRN complex which is recruited at the sites of DSBs [46]. Following ATM activation, 53BP1 interacts with its effector molecule, RIF1, which is recruited at the sites of DSBs and is also antagonized by BRCA1 and CtIP leading to the induction of the appropriate DNA repair pathway(s) [47]. Moreover, hyperthermia has been shown to delay the employment of 53BP1 in MRN complex formation [23] whereas activation of ATM is decelerated after heat exposure [38]. Furthermore, data from other reports have associated the activation of ATM with generation of oxidative stress as it was shown that ROS can oxidize the disulfide bond between the monomers of the ATM dimer without the presence of MRN and thus causing directly its activation [48]. To this end, experiments from different studies showed that, after exposure to hyperthermia, there were increased levels of ROS and 8-oxoguanine generation, a finding that could contribute to the activation of ATM [49] and its ability to directly phosphorylate Chk2 [50]. Overall, it can be suggested that exposure to hyperthermia can delay the activation of ATM-Chk2 caused by oxidative stress induction which can potentially lead to formation of DSBs.

Moreover, another molecule associated with the DNA damage response pathways is the E2F1 transcription factor. E2F1 is phosphorylated at Ser31 by ATM or ATR leading to its stabilization, in response to DNA damage, which is then recruited at DNA damage sites where it enhances DNA repair [51]. Other studies have implicated its role in apoptotic induction by activating p53 family dependent and independent pathways as well as DDR. However, there are reports supporting its pro-apoptotic role in different types of cancer. Such

contradictory observations regarding E2F1's behavior may as well depend on different cancer types and variation of apoptotic stimuli so its pro- or anti-apoptotic role in every case requires well designed and in depth analyses [52]. Regarding E2F1's role upon heat application, there has been proof from a recent research report utilizing electro-hyperthermia in human glioma cell lines as well as an *in vivo* mouse model that hyperthermia resulted in the up-regulation of E2F1 followed by apoptotic activation [53].

Ultimately, the effects of hyperthermia on DNA damage response pathways can translate in terms of sensitizing cancer cells against other means of therapy in order to potentiate their effectiveness. For instance, several studies using anti-mitotic drugs such as paclitaxel, nocodazole and Aurora A in combination with hyperthermia have shown a significant increase in cytotoxicity together with mitotic catastrophe [54], an event that has also been observed when HeLa S3 cells were exposed at 41.5°C prior to being subjected to irradiation [55]. In conclusion, ATM and ATR are the main molecules regulating the cell cycle checkpoints following hyperthermia-induced DNA damage (e.g. DSBs or SSBs), an observation which further contributes to the activation of the DDR pathway.

According to a recent report, there is a second checkpoint following DDR activation which involves the induction of the tumor-suppressor ARF. Data from *in vivo* experiments involving different mouse cancer models and human epithelial samples demonstrated that ARF activation occurs later than the DDR one and it is also rare. The delayed induction of ARF has been suggested to be due to the requirement of a higher threshold of oncogenic load compared to DDR [56]. Results from another study outlined the relationship between ARF and ATM, as it was shown that there is an up-regulation of ARF in the case of inhibition of ATM or loss of its expression, thus supporting the importance of ARF's role as a complementary anticancer barrier against tumor progression [57]. In addition to its previously described role in cancer development, there has been evidence that ARF can also be activated

by oxidative stress [58]. Although there is lack of studies investigating into how ARF can be affected by hyperthermia, there is evidence that the loss of ARF results in enhanced resistance to cell death induction in response to heat treatment in HeLa and H1299 cells [59]. Therefore, given the evidence suggesting that ATM induction is decelerated in response to heat, it can be speculated that this could cause the triggering of ARF which in turn is strongly activated when ATM is lost [57].

### **3. Hyperthermia and DNA repair mechanisms**

In general, cells have developed a number of different mechanisms to ensure genome integrity as well as to repair damaged DNA. Briefly, there are six main DNA repair pathways including i) base excision repair (BER), ii) mismatch repair (MMR), iii) nucleotide excision repair (NER), iv) translesion DNA synthesis (TLS), v) non-homologous end joining (NHEJ) and vi) homologous recombination (HR) [60,61]. Moreover, excision repair can be divided into three categories namely BER, MMR and NER [62]. In this section, we will describe how hyperthermia can influence these DNA repair pathways (Fig. 3).

#### **3.1 Base Excision Repair (BER)**

BER is considered to be the primary DNA repair cascade which is responsible for the correction of base lesions resulting from oxidative, alkylation, deamination and depurination / depyrimidination damage. A number of different proteins take part in this pathway including AP endonucleases, end processing enzymes, DNA polymerases, glycosylases and ligases. The pathway is initiated when DNA glycosylases recognize and remove inappropriately paired bases thus forming apurinic/apyrimidinic (AP) sites. Subsequently, AP endonucleases (in particular APE1) process these sites and in doing so recruit DNA polymerases which repair resulting SSBs (through short-patch or long-patch BER) and together with DNA ligases seal

the corresponding DNA strand. Overall, BER can be described as a complex process of paramount importance for maintaining the removal and restoration of any damaged bases that can potentially result in mutations [63].

Several studies have demonstrated the effect of hyperthermia on BER by various means [64,65] including its effects on the activity of i) DNA polymerases (e.g. reduction of the activity of DNA Pol $\beta$ ) [66], ii) DNA glycosylases (e.g. inactivation of 8-oxoguanine DNA glycosylase; OGG1) [67] and iii) DNA repair proteins (e.g. induction of XRCC1) [68,69]. Finally, other groups have focused on investigating the role of AP endonucleases in hyperthermia induced radio-sensitization however, after siRNA-induced knocking down of APE1, in HeLa cells, there were not any observed potentiation effects [70].

### **3.2 Nucleotide Excision Repair (NER)**

NER plays an equally critical role in the cell's repair system as it is responsible for removing DNA damage induced by ultraviolet radiation (UVR). The most significant lesions removed by this pathway are pyrimidine dimers and 6,4-photoproducts. Moreover, it has been argued that this pathway may be also contributing in the observed resistance to platinum-based chemotherapy. NER can be divided into two pathways namely global genome NER (GG-NER) and transcription-coupled NER (TC-NER). As implied by their names, GG-NER is a process in charge of inspecting and eliminating DNA lesions throughout the whole genome whereas TC-NER is specifically activated to repair lesions on the transcribed strands of transcriptionally active genes. These two cascades vary regarding the recognition step of the damaged DNA but they follow the same processes for the excision, repair and ligation steps in repairing a DNA lesion [71].

Some studies have argued that hyperthermia is able to influence NER by means of enhancing the sensitivity to platinum-based drugs when combined with exposure to elevated

temperatures. Platinum-containing anticancer drugs like cisplatin, carboplatin, oxaliplatin, etc. have the ability to bind to DNA and result in the formation of inter-strand crosslinks, while defects in replication and transcription pathways appear to enhance the sensitivity to cisplatin and mitomycin C treatments [72]. To these ends, NER appears to be the primary pathway activated for the removal of pyriplatin-DNA adducts formed in platinum-based treatments [73]. On the contrary, work by other groups (in utilizing normal human fibroblasts along with NER deficient ones) have shown no beneficial effect of hyperthermia in enhancing sensitivity to cisplatin, regardless of NER deficiency or not [74] suggesting the contribution of other DNA repair pathways, such as TLS [72]. Finally, *in vivo* studies utilizing a murine metastatic ovarian cancer model have also shown that hyperthermia can contribute to the sensitization of tumors against cisplatin by inhibiting NER [75].

### **3.3 Mismatch Repair (MMR)**

MMR is defined as a system which is able to recognize and repair inaccurate insertions, deletions and inappropriate incorporations of bases which have escaped the proofreading activity of replication enzymes. This pathway consists of i) the recognition of the mis-paired base(s), ii) the excision of the erroneous part of the strand and creation of a gap at that location and finally iii) the synthesis of the correct DNA strand. Numerous proteins participate in completing all these steps including those of MutS, MutL, MSH2, MSH3, MSH6, MLH1, MLH3, PMS1, PMS2, Exo1, DNA polymerase, PCNA, RPA, DNA ligase, etc. MMR deficient cells have been shown to incur genome instability and more specifically present microsatellite instability, a common characteristic of cancer cells as it results in elevated mutation levels [76]. Surprisingly, MMR deficient tumor cells appear to demonstrate resistance to cisplatin, carboplatin, busulfan, procarbazine, 6-thioguanine, etoposide and



doxorubicin, an observation that could imply the involvement of MMR in triggering other signaling pathways such as apoptosis [77].

There seems to be limited literature concerning the potential impact of hyperthermia on MMR. A recently published paper looked into possible interactions between heat-shock proteins (HSPs) (e.g. HSP27 and HSP72) and those involved in MMR (e.g. MLH2 and MSH2) by utilizing human MMR<sup>-</sup> and MMR<sup>+</sup> colon adenocarcinoma cell lines exposed to hyperthermia prior to treatment with cisplatin. Results showed that MLH2 and MSH2 interact with HSP27 and HSP72 and that hyperthermia leads to cell cycle arrest as well as enhancing the expression rates of p72 and the sensitivity to cisplatin treatment in MMR<sup>+</sup> cells [78]. Another report published by the same group had also demonstrated that hMLH1 and hMSH2 were translocated from the nucleus to the cytoplasm at 41°C and 42°C while exposure to these temperatures was also accompanied by an elevation in the expression rates of HSP27 and HSP72 in human colorectal cancer cell lines [79]. In addition, the co-localization of HSP27 and HSP72 with hMLH1 and hMSH2 has also been reported in peripheral blood lymphocytes from healthy individuals exposed to hyperthermia and combined with cisplatin treatment [80]. Finally, another report associated HSPs with temozolomide resistance in human glioma cells as there appears to be an interaction between HSPB1 and MSH2 thus affecting the MMR pathway [81].

In conclusion, the above-mentioned excision repair cascades play a significant role in repairing DNA damage induced by various chemotherapeutic agents including cisplatin, adriamycin, bleomycin, melphalan, cyclophosphamide, nitrosoureas, taxanes, etc. used for the treatment of various cancers. The capacity of hyperthermia to potentiate the action of chemotherapeutic drugs contributes to improved and more effective therapeutic strategies in terms of using lower but equally effective drug doses, a fact which is essential in drug-induced cytotoxic side effects and the overall benefit to patients' health outcome [82].

### 3.4 Translesion DNA Synthesis (TLS)

This can be described as a DNA damage tolerance process which can guide the replication of DNA lesions such as thymine dimers or AP sites. This process involves the activation of alternative translesion polymerases (e.g. DNA polymerase IV or V) through PCNA and not regular polymerases. The DNA replication process is stalled at the replication forks due to DNA adducts which can recruit polymerases at that location. Activation of specialized polymerases repairs the lesion, while Pol $\zeta$  might be needed if a mismatch has to be extended. Finally, PCNA controls the induction of the proper polymerases for carrying on DNA replication. A significant number of TLS polymerases have been identified but their role and mechanisms of action are not fully elucidated yet. However, there is compelling evidence that cancer cells exploit TLS polymerases due to their lower fidelity compared to DNA replication polymerases in order to increase the frequency of mutations [83,84].

Apparently, there is a lack of reports on the potential impact of hyperthermia on TLS. However, there is evidence that HSP90 can regulate the interaction between Pol $\eta$  and PCNA, an important event for the localization of the enzyme on the site of the stalled replication forks [85]. Furthermore, data from a following study has shown that HSP90 acts as a regulator of REV1 (a molecule responsible for causing mutations in error-prone TLS) and also interacts with Pol $\eta$  [86]. Increased expression levels of Pol $\eta$  have been found in ovarian CSCs thus implying the involvement of a Pol $\eta$ -mediated TLS which seems to contribute to their observed resistance in cisplatin treatment [87]. Taken together, it can be speculated that hyperthermia can influence TLS as there is evidence for the existence of interactions between HSPs and molecules participating in TLS however, more research in this area is required.

### 3.5 Non-homologous End Joining (NHEJ)

This is one of the DNA repair pathways responsible for repairing DSBs and it does not require a homologous template. Instead, NHEJ is depended on short homologous DNA sequences, the micro-homologies, which are usually present in single stranded overhangs at the end of DSBs. NHEJ has been divided into two sub-pathways namely i) the canonical NHEJ (c-NHEJ) and ii) the alternative NHEJ (alt-NHEJ). The alt-NHEJ involves different enzymes for its completion and is generally considered to be more error-prone than c-NHEJ [88,89].

There is conflicting evidence regarding whether NHEJ is affected by hyperthermia or not. Early studies indicated that DNA repair pathways might be affected by hyperthermia in a series of radio-sensitization experiments using wild type and repair deficient Xrs-5 cells. In these studies, it was found that wild type cells were sensitized to irradiation in greater levels compared to Xrs-5 cells when exposed to hyperthermia at 43°C and/or 45°C [90,91]. Following studies determined the lack of the active Ku protein in Xrs-5 cells, an observation indicating that the DNA repair cascade affected by hyperthermia was NHEJ [92]. It was later suggested that hyperthermia can act as a radio-sensitizer by interfering with the repair of DSBs as it may have an impact on c-NHEJ, alt-NHEJ and HR collectively [93]. Recent work showed that exposure to hyperthermia can restrict the activity of DNA-PKs in addition to reducing protein levels of KU70, KU80, BRCA1 and 53BP1 with the first two in greater extent [94]. Finally, given that various chemotherapeutic agents have the ability to trigger the induction of DSBs, inhibitors of DNA ligases and/or DNA-PKs appear to sensitize tumor cells to chemotherapeutic drugs thus providing alternative therapeutic strategies which potentially can be more effective [95,96]. To this end, it can be speculated that exposure to hyperthermia in combination with various inhibitor drug molecules might have a significant impact on the NHEJ pathway and thus provide in potentiating their therapeutic effectiveness.

### 3.6 Homologous Recombination (HR)

This is a DNA repair pathway that requires a homologous template (unlike NHEJ) for repairing DSBs and because of such requirement HR is thought to occur during the S and G2 phases of the cell cycle [97].

Initial reports utilizing cell lines deficient or proficient in HR investigated its potential relationship with hyperthermia and observed that this DNA repair pathway might also be a key target for the action of elevated temperatures [65,98]. More specifically, observations by a group using chicken DT40 cells deficient in HR appeared to be more sensitive to irradiation in response to hyperthermia exposure [99]. Furthermore, more recent reports have revealed the ability of mild hyperthermia (42°C to 42.5°C) to inhibit HR in experiments utilizing mouse embryonic stem cells proficient or deficient in HR. According to these results, HR deficient cells were more resistant to radio-sensitization compared to the wild type (proficient) cells (after being exposed to hyperthermia) suggesting RAD51 (involved in the search for homology and strand pairing stages of the HR process) being the principal targeted molecule. In addition, hyperthermia at 41°C appears to cause partial degradation of BRCA2 as well as inhibition of HSP90 (known to interact with BRCA2) thus resulting in HR inhibition and consequently increased sensitivity to the effects of PARP-1 inhibitors and irradiation [100]. Moreover, it was found that hyperthermia can cause the inactivation of the Replication Protein A (RPA) [101] while it can also affect the formation and interactions among the proteins of the MRN complex, depending on the severity of the hyperthermia exposure [102]. Finally, MRE11 (a double-strand break repair protein) and RAD51 recruitment (at replication forks) was shown to be also inhibited after a combinational treatment protocol consisting of hyperthermia and gemcitabine [103]. Overall, as in the case of NHEJ, combined treatment of hyperthermia with chemotherapeutic agents can enhance the

therapeutic outcome of the treatment protocol by means of targeting the HR pathway in addition to other potential candidate DNA repair cascades [96].

#### **4. Hyperthermia in combination with DNA damaging treatments in *in vivo* studies**

Over the last few years, there has been promising evidence of inhibition of tumor growth and suppression of metastasis in several *in vivo* studies utilizing heat elevation in combination with various DNA damaging agents. More specifically, a recent study utilizing a human colon adenocarcinoma (HT-29) model, in nude mice, demonstrated that the use of Dbait (a DNA repair inhibitor) combined with radiofrequency ablation (at mild hyperthermic temperatures) led to significant reduction in tumor size or even complete tumor regression. In addition, it was shown that Dbait led to the phosphorylation of H2AX by inducing the activation of DNA-PK (in orthotopically grafted liver tumors) thus supporting its potential role in effectively treating tumor metastasis by interacting with DNA repair cascades [104]. Moreover, data from another study demonstrated that the utilization of doxorubicin-loaded DNA wrapped gold nanorods together with photothermal ablation in a mouse breast cancer (4T1) model resulted in tumor growth reduction and suppression of lung metastasis [105]. Finally, in another study utilizing a human ovarian (A2780/CP70) cancer model in nude mice, the use of hyperthermic sodium arsenite in combination with cisplatin administration enhanced cisplatin's therapeutic effectiveness by inhibiting NER, while activating MMR and increasing its accumulation in the tumors [75]. Collectively, an increased number of *in vivo* studies confirm the beneficial effects of hyperthermia in combined treatment protocols and thus can further support and promote the application of adjuvant hyperthermia-based therapeutic protocols in future clinical trials.

## 5. Hyperthermia in clinical practice

Since the 1970s and 80s, hyperthermia has been used as an adjuvant therapy in clinical practice for the treatment of various cancer types. Although, its application was quite restricted at the beginning (probably due to earlier technical difficulties in delivering heat on the tumor site) its beneficial effects were evident from early studies [106,107]. A great number of randomized phase II and III clinical trials utilizing hyperthermia have outlined its contribution in potentiating the efficiency of irradiation in treating breast [108], cervical [109], lung [110], head and neck [111], melanoma [112], gastrointestinal [113] and sarcoma cancers [114]. Furthermore, hyperthermia combined with several chemotherapeutic agents has been the subject of numerous phase II and III clinical trials. For instance, a recent clinical trial involved the application of a treatment protocol based on hyperthermia combined with cisplatin and paclitaxel for treating advanced ovarian cancer [115]. Another example of an adjuvant hyperthermia therapeutic protocol included treatment with etoposide, ifosfamide and doxorubicin for treating soft tissue sarcoma [116]. In fact, it is very difficult to provide a detailed list of clinical trials utilizing adjuvant hyperthermia with chemotherapeutic drugs and/or irradiation due to their vast number. However, only recently, an overview on the enhanced effectiveness of a wide variety of DNA damaging chemotherapeutic in combination with hyperthermia has been published which extensively covers this topic [117]. Obviously, it is an undeniable fact that the wide range of chemotherapeutic agents used in clinical practice today, when combined with hyperthermia, can interact with DNA repair pathways thus providing for an improved and more efficient therapeutic outcome in cancer patients.

## 6. Conclusions

Over the past decades, hyperthermia has evolved into a continuously more exploit and effective therapeutic means in treating various types of cancer. Heat elevation is applied

as a complementary therapy along with chemotherapy and/or radiation therapy by several clinics around the world. Tumor cells are characterized by genomic instability, increased mutation rates and ability to escape cell death induction all of which aid in maintaining endless cell proliferation. For this reason, targeting their DNA is a major therapeutic strategy for their elimination. To this end, hyperthermia contributes directly (Fig. 1) and/or indirectly (Fig. 2) in inducing DNA damage in addition to interacting and interfering with various DNA repair cascades (Fig. 3) all of which are important in eliminating cancer cells. On the other hand, it is noteworthy that during the last few years there has been a growing interest in investigating the potential role of hypothermia during radiotherapy in terms of protecting normal tissue. There is evidence showing that hypothermia treatment (13°C) combined with irradiation results in radioprotection of immortalized foreskin fibroblast cells and human lymphocytes which might be explained, partially, by the observed lack of DSBs rejoining and lower levels of phosphorylated H2AX [118]. This suggests that hypothermia is responsible for the suppression of DNA repair pathways (in response to irradiation) perhaps in a manner comparable to that observed for hyperthermia although acting in an opposite direction. In any case, it is rather inconclusive if the effects of hypothermia and hyperthermia are mediated by their ability to suppress DNA repair pathways but nevertheless suggest that they may be important as potential underlying mechanisms. Consequently more elaborate studies are needed in order to determine their precise involvement.

Despite the large number of research reports investigating the effects of heat elevation on inducing the DNA damage response and interfering with various DNA repair pathways, the underlying mechanisms are not yet completely elucidated. Advanced technology for heat delivery as well as recent developments in molecular biology methodologies, have allowed for a more focused and effective approach in the treatment of various types of tumors with the potential to be more personalized. Moreover, the effects of

improved irradiation and chemotherapy-based therapeutic protocols have been undoubtedly enhanced by the adjuvant application of hyperthermia as they appear to act synergistically in DNA response and repair pathways thus leading to the elimination of tumor cells. Although, there is a long way before we reveal the underlying mechanism(s) by which hyperthermia exerts its therapeutic effectiveness, nevertheless it has been documented as a promising approach for potentiating the therapeutic outcome of other modalities in cancer treatment. This, in turn, is of paramount importance as more efficient targeting drug compounds are designed with the potential to be more effective in treating various forms of cancer and potentially add value in the context of improving the patients' quality of life.

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## **8. Conflict of Interest statement**

The authors declare that there are no conflicts of interest



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**Figure Legends:**

**Figure 1:** The direct effects of hyperthermia. The generation of DNA breaks, SSBs and DSBs, formation of  $\gamma$ H2AX foci and ATM auto-phosphorylation result in DDR induction while the observed reduction in DNA polymerases' and topoisomerases' activity is responsible for delayed DNA replication thus affecting DNA repair.

**Figure 2:** The indirect effects of hyperthermia. Heat elevation can lead to increased generation of ROS therefore inducing oxidative stress which will eventually cause DNA damage. Furthermore, hyperthermia is also able to cause cell cycle arrest and cell cycle checkpoint activation which in turn results in the induction of ATM or ATR. The observed retardation of DNA replication processes is also attributed to heat elevation and results in increased DNA damage levels. Finally, the up-regulation of E2F1 in response to hyperthermia has been implicated in the activation of cell death pathways.

**Figure 3:** Overview of the different DNA repair mechanisms induced by hyperthermia

Figure 1

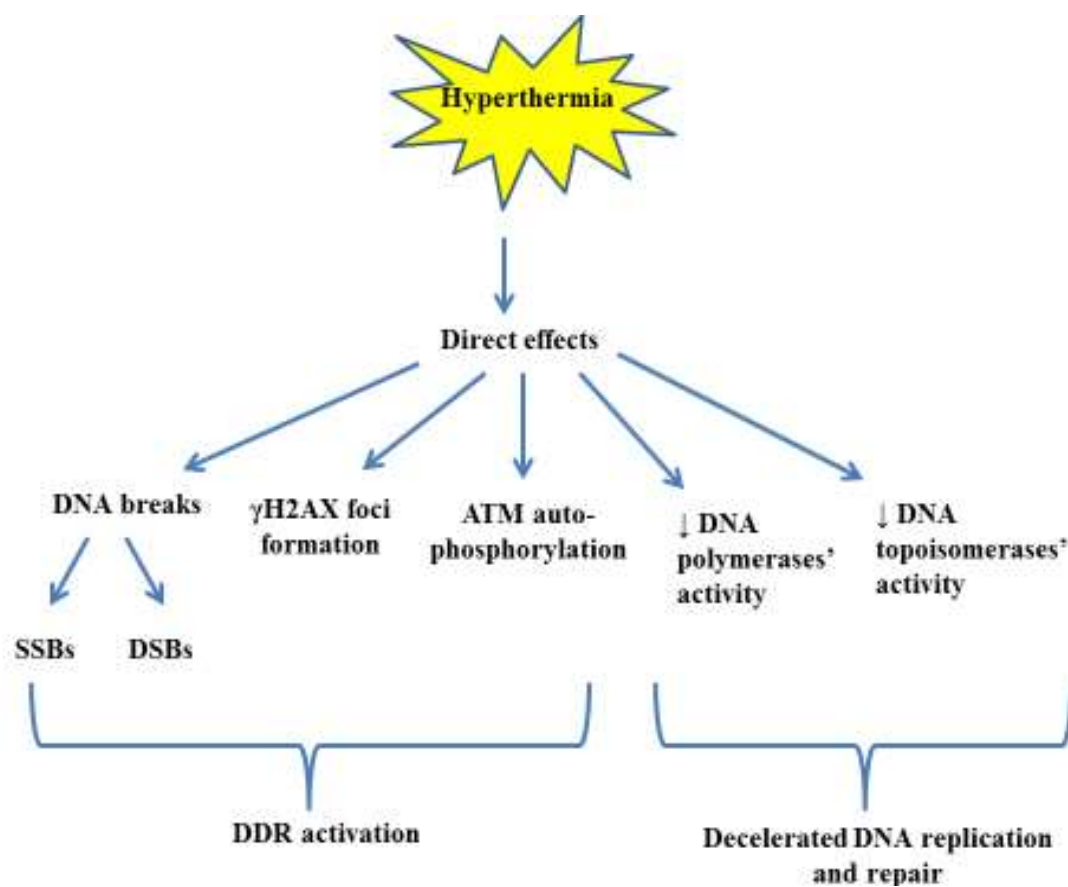


Figure 2

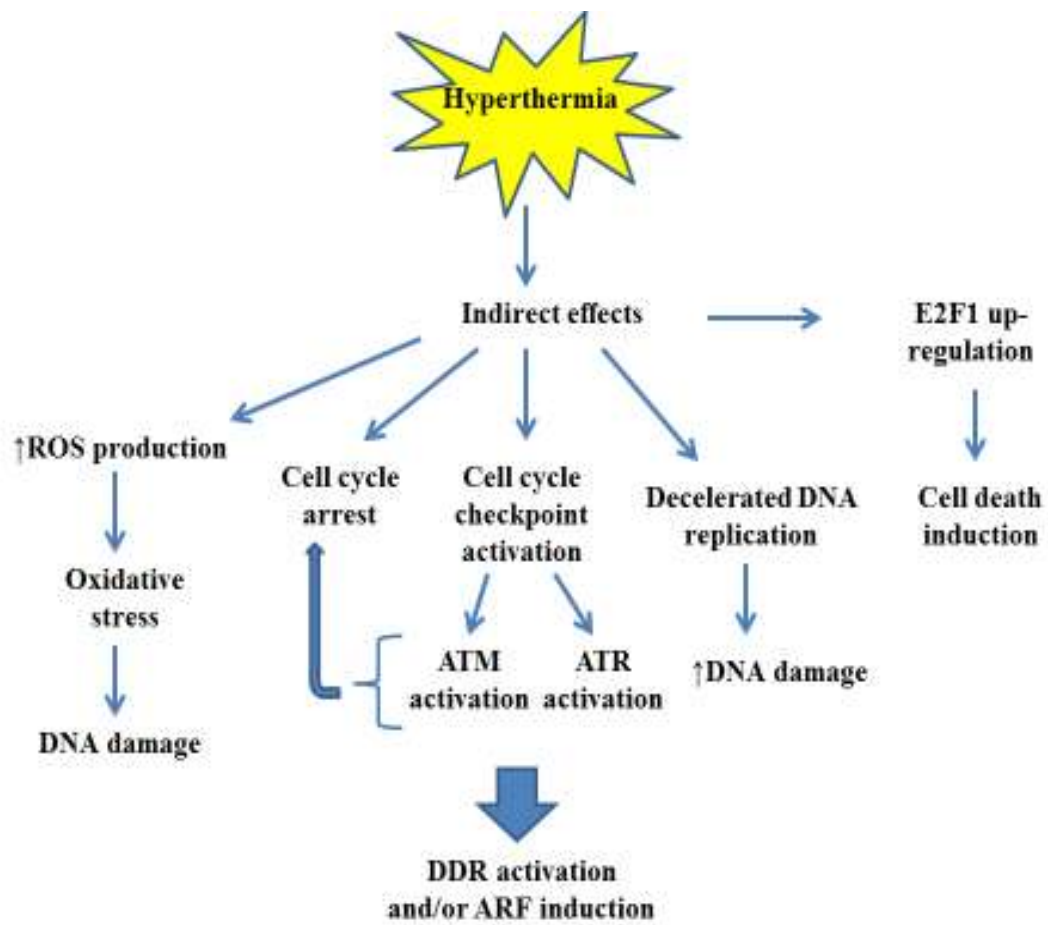


Figure 3

