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Oxidative Stress in Hadrontherapy

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Abstract

Conventional radiotherapy has shown its efficiency since decades with large progresses during the 1990s. However, for 15–20% of treated patients, there is no prognosis improvement either due to tumor radiation resistance and/or to side effects on normal tissues representing the limiting dose given during a radiotherapy protocol. A new modality of radiation therapy has emerged representing a technological breakthrough: hadrontherapy. This regroups mainly proton and carbon ion therapy. Dose deposit is in favor of hadrons compared to photons as it occurs at a precise depth in human body sparing upstream and downstream normal tissues. Mechanisms of action of photons and hadrons are different. When photons mainly act by water radiolysis—producing e^-_{aq}, $H^\bullet$, $^\bullet OH$, H_2O_2, $O_2^{\bullet-}$…, carbon ions and protons mainly act by direct effects, i.e. by direct transfer of ion energy to biological macromolecules. Moreover, efficiency of carbon ions is considered threefold higher (1.1 for protons) than X-rays in killing tumor cells, whereas it is considered lower for normal cells. These findings suggest strong advantages of hadrontherapy compared to conventional radiotherapy. However, some recent studies tend to show a stronger increase in oxidative stress in normal cells after protons or carbon ions than X-rays.

Keywords: hadrontherapy, oxidative stress, carbon ions, protons, DNA damage, tumor killing efficiency, normal tissue toxicity, senescence, inflammation

1. Introduction

Oxidative stress is of major interest in killing tumor cells. In this way, radiation therapy is one of the most used modality for cancer treatment (**Figure 1**). Ionizing radiations lead to the production of deleterious reactive oxygen species that overcome antioxidant systems resulting in tumor cell death. On the 14 million of new cancer cases each year in the world, about half of them will benefit from this treatment [1].
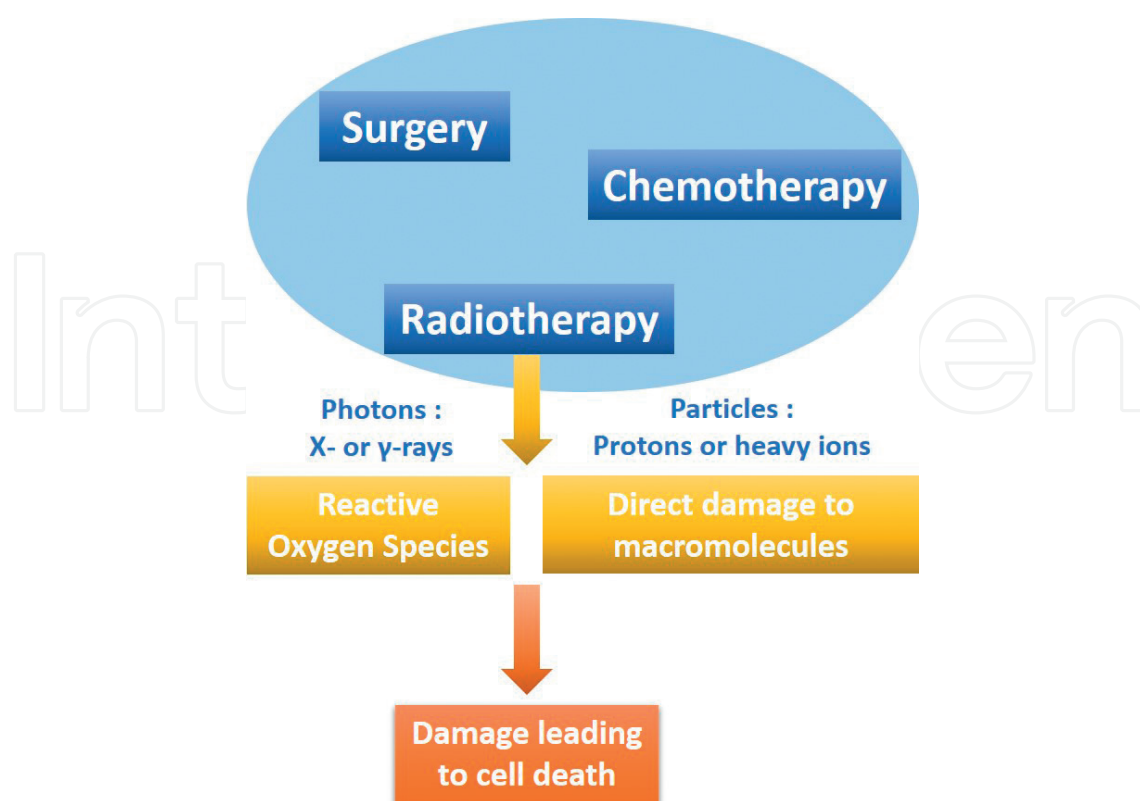
Figure 1. Cancer treatment: focus on radiotherapy.

Conventional radiotherapy—by photons (γ- or X-rays)—has known a revolution since the 1990s, mainly thanks to progresses in imagery, computer sciences and robotics. In this way, new modalities of radiation therapy occurred: intensity-modulated radiotherapy (IMXRT), image-guided radiation therapy (IGRT) and respiratory-gated radiotherapy: the 4D radiotherapy. These new kinds of treatments allowed to overcome the main difficulties encountered in conventional radiotherapy: the exponential dose deposit which leads to an overdose in normal tissues upstream and downstream from the tumor (**Figure 2**, left panel) [2].

In parallel, therapy by accelerated hadrons was developed since the 1950s (Berkeley, United States). Hadronic particles regroup neutrons, protons, pions, antiprotons, helium, lithium, boron, carbon and oxygen ions. The major interest of protons and heavy ions (mass greater than helium) lies in the profile of dose deposit: the Bragg peak (**Figure 2**, right panel). Contrary to conventional radiations, dose distribution is in favor of normal surrounding tissues as the maximum of dose is deposited at a precise depth in the matter with a larger peak for protons than for carbon ions. However, a plateau phase does exist upstream from the peak, resulting in a small proportion of dose deposition in normal tissues preceding the tumor, as well as a fragmentation tail downstream from the peak (except for protons which cannot fragment in smaller particles). Moreover, in the case of heavy ions, their fragmentation when encountering matter lead to secondary particles, which properties are different in terms of LET (linear transfer energy) and biological effects. In addition, to treat the whole tumor volume, hadron beam energy and direction are modified to spread the peak: SOBP, Spread Out Bragg Peak (**Figure 2**, right panel). This leads to an addition of plateau phases as well as fragmentation
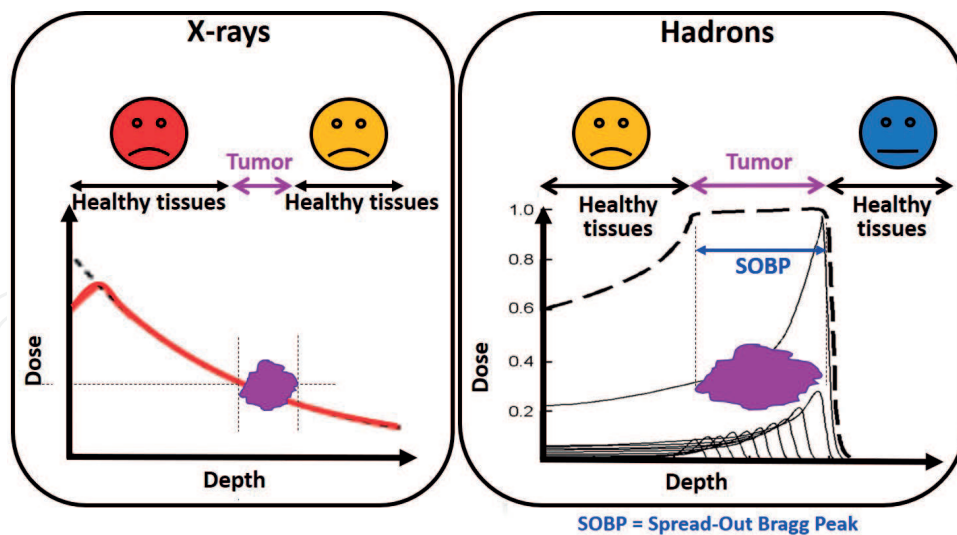
Figure 2. Dose deposit after X-rays in comparison to hadrons. Left panel: Dose deposit of X-rays according to depth in human body. Right panel: Dose deposit of hadrons according to depth in human body. Bragg peak: Continuous black line. SOBP: Dotted line.

tails. In this way, normal surrounding tissues received a percentage of dose that could be non-negligible according to the tumor size and localization.

Radiations lead to a wide range of oxidative damage to DNA, lipids and proteins. Effects of photons were widely studied *in vitro* and *in vivo* since decades. When photons mainly act by indirect effects, i.e. water radiolysis—producing e^-_{aq}, $H^\bullet$, $^\bullet OH$, H_2, H_2O_2, H^+, OH^-, $O_2^{\bullet-}$…, carbon ions and protons mainly act by direct effects, i.e. by direct transfer of ion energy to biological macromolecules.

We propose to develop involvements of oxidative stress in: (i) tumor cell killing efficiency of hadrontherapy and (ii) side effects of hadrontherapy—secondary tumors and normal tissue injury.

2. Oxidative stress and tumor cell killing efficiency of hadrontherapy

The main advantages of the use of hadrons in comparison with photons are their superior dose localization, their efficiency against radioresistant and hypoxic tumors and the ability to shorten treatment planning.

2.1. Interest of protons and carbon ions in clinics

Due to their high charge, heavy ions lead to concentrate ionizations when they cross matter. These concentrate ionizations result in concentrate oxidative damage. On the contrary, when photons (low LET) encounter matter, they produce low ionization densities. Tumor cell killing is more efficient with hadrons as, for example, clusters of DNA damage are produced leading to difficult DNA repair in comparison with photons producing more easily repaired SSB (single-strand breaks). Efficiency—RBE for Relative Biological Efficiency—of carbon ions

is considered twofold to threefold higher (1.1 for protons) than X-rays in killing tumor cells. These RBE are calculated for a percentage of clonogenic survival of 10%. However, experiments leading to these values were performed under a broad range of conditions, among other things: LET or cell cycle phase—cell irradiation at confluence stage or during exponential phase. In this way, higher RBE than 3 were found—up to 5, for example, 3.3 for normal human skin fibroblasts exposed at confluence stage to mimic skin physiology to carbon ions in the plateau phase before Bragg peak as it would be the case during radiotherapy [3]. Biological interactions of protons and carbon ions being a lot more complex than photons, and to improve hadrontherapy, there is a need of a better knowledge of biological effects, at early and late times, of hadrons according to LET, fractionation, cell type, oxygenation, cell cycle phase, etc. (for review [4]). Due to the favorable dose deposit profile, this kind of therapy is recommended for unresectable and radioresistant tumors. Until now, more than 110,000 patients have been treated by proton therapy and 15,000 patients by carbon ion therapy.

Concerning protons, numbers of pediatric tumors were treated by protons as the dose deposit should be favorable for normal surrounding tissues: medulloblastoma [5, 6], rhabdomyosarcoma [7, 8], craniopharyngioma [9], etc. There is a trend to extend the indications for proton therapy from already treated skull base [10, 11] and brain [12–14] tumors to prostate [15–17], lung [18–20], head and neck (for review, [21]), gastrointestinal (for review, [22]) and breast [23] cancers. Compared to conventional radiotherapy, proton therapy obtained the same results in terms of tumor local control (for review, [24]). The superiority of protons is still discussed, except in large ocular melanomas, chordomas and chondrosarcomas [25].

The main experienced facilities providing carbon ion beams and treating a big number of patients are: NIRS (Japan) and GSI and then HIT (Germany). The main indications were, as for protons, not only pediatric cancers but also bone and soft tissue sarcomas; head and neck cancers; pancreas, prostate and cervix cancers; hepatocellular carcinomas and glioblastoma (for review, [26–28]). Carbon ion therapy present significant advantages, but, due to a lack of available data in the literature, clinical evidences are still lacking.

2.2. DNA damage and repair, mitotic catastrophe

Hadrons are considered as acting mainly by direct effects. Carbon ions are particularly deleterious in terms of cell survival, viability and apoptosis, even on very radioresistant tumor cell lines [29, 30]. This efficiency to kill tumor cells could come from the type of damage produced by carbon ions: DSB (double-strand breaks) and clustered DNA damage considered as difficult to repair. Clusters of damage could be a criterion explaining ion irradiation efficiency as it was shown that cluster number increases with LET. However, Hada et al. [31] have shown that DNA damage (DSB, abasic sites, oxidized bases) number decreased in genomic DNA irradiated at high LET and DSB was more frequent than other damage after charged particles, even low-LET protons, than after X-rays. In the same manner, Heilmann et al. [32] demonstrated that carbon ion irradiation (LET from 14 to 400 keV/μm) did not generate more DSB than X-rays (kV) with a maximum of about 38 DSB/Gy/cell. A possible explanation for the strong RBE of carbon ions could be related to DNA damage repair. Moertel et al. [29] showed that residual DSB were more numerous in ion-irradiated human glioblastoma cells than in

X-ray-irradiated cells. Moreover, Weyrather et al. [33] highlighted that carbon ion RBE was related to cell repair capacity. The role of HR (homologous recombination) was highlighted after proton irradiation as deficiency in this pathway leads to a sensitization of cells to protons [34]. DNA repair by the Ku-dependent NHEJ (non-homologous end joining) pathway was shown as inhibited by high-LET irradiation [35]: the yield of DSB should be the same after low- or high-LET irradiation but high-LET induced smaller fragments inhibiting the efficient binding of Ku to DSB fragment. However, there are reports of a primordial role of NHEJ after carbon ions as inhibition of DNA-PKcs led to a sensitization of cancer cells to carbon ions [36]. Recent report pointed out another response to DNA damage after carbon ion irradiation: mitotic catastrophe. Kobayashi et al. [37] demonstrated that mitotic catastrophe phenomenon was induced in a larger manner after carbon ions than after X-rays in 20 human cancer cell lines, whereas apoptosis and senescence were unchanged between both radiation types.

2.3. Oxygen effect and carbon ions

Hypoxic tumor cells are reoxygenated during radiotherapy treatment, and this reoxygenation plays an important role on treatment efficacy [38]. Hypoxic tumors resist to X-rays, whereas carbon ion exposure remains efficient [39]. The same study showed that this could come from a faster reoxygenation of tumors after carbon ion irradiation compared to X-rays. This was confirmed by Oya et al. [40] and also by Fukawa et al. [41] by pO2 measurements in mouse fibrosarcomas. Recently, Wozny et al. [42] have shown that hypoxia-induced factor HIF-1α, whose role was demonstrated in radioresistance to conventional radiotherapy, is expressed earlier in carbon irradiated cancer stem cells—subpopulation of head and neck squamous cell carcinoma—localized in tumor hypoxic areas. In the presence of oxygen, ROS quantity increases leading possibly to a major oxidative stress and so to a stronger attack of biological macromolecules. Oxygen effect could play a major role in the difference observed between carbon ion and photon responses as hypoxia leads to a decrease in DSB repair capacity [43]. Hirayama et al. [44] observed a decrease in DNA damage after hypoxia but this was much less significant after carbon ion than after X-ray irradiation. Moreover, the same study demonstrated that repaired DSB percentage was unchanged after carbon ion irradiation in hypoxic conditions, which is not the case for X-rays. The authors concluded that DSB repair plays an important role in oxygen effect as this effect was decreased after carbon ion irradiation compared to X-rays. This could be related to a stable effect of oxygen on DSB during the time after carbon ion irradiation, whereas it decreases after X-ray irradiation.

2.4. Role of oxidative stress in hadrontherapy efficiency

Studies are controversial concerning protons. The use of edaravone, a radical scavenger, did not decrease DNA DSB formation in MOLT-4 tumor cells after protons as it was the case for X-rays leading to conclude that radical-induced indirect DNA damage was lower with protons than with X-rays [45]. However, Baran et al. [46] showed that proton irradiation led to a disruption of the electron flow in the complex I of the mitochondrial respiratory chain in human leukemia Jurkat T cell, and the use of antioxidants in HeLa cancer cell line allowed an attenuation of the enhancement of radiation-activated gene expression [47].

Concerning carbon ions, studies performed at high radiation doses (30 Gy) on murine squamous cell carcinoma and fibrosarcoma transplanted in mouse allowed to provide evidence of a strong upregulation of stress-responsive and cell communication genes after carbon ion irradiation compared to γ-rays [48]. Moreover, glutathione depletion in human squamous cell carcinoma cell lines potentiates the effects of carbon ion irradiation [49]. In this way, heavy ions do not act only by direct interaction with biological macromolecules but also by an induction of oxidative phenomena.

3. Oxidative stress and side effects of hadrontherapy

Radiotherapy aims to destroy cancer cells by the use of photons, protons or heavy ions. But this is a double-edged sword as it can also kill normal cells. Two types of side effects can appear: deterministic (pneumonitis, gastrointestinal or cutaneous syndrome, etc.) or stochastic (carcinogenesis and genetic effects). Indeed, dose deposit is exponential for photons so that the maximum of the dose is given at the entrance in the body before reaching tumor. In this way, normal tissues—present upstream and downstream from the tumor—receive ionizing radiations leading to ROS (reactive oxygen species) production. When normal cells are unable to detoxify these ROS, there is an imbalance leading to oxidative stress. Signaling pathways leading to inflammation maintain this process, therefore participating to side effects on normal tissues. It is considered that 5–10% of the general population exhibit acute or late adverse effects after radiotherapy. For example, pneumonitis is observed in 5–15% of patients irradiated for breast, lung and mediastinal tumors [50]. By the use of hadrons, organs at risk present around the tumor could be spared, and biological efficiency is considered higher in tumors than in normal tissues. In this way, treatment time could be shortened by hypofractionation of the total radiotherapy dose: 3 weeks compared to 6–7 weeks.

3.1. Toxicity encountered in patients after proton or carbon ion therapy

Toxicities of radiation therapy can not only occur at skin level (dermatitis, telangiectasia, etc.), cardiovascular and pulmonary level (pneumonitis, cardiovascular disease, etc.), gastrointestinal level (xerostomia, mucositis, esophagitis, enteritis, proctitis, emesis) and genitourinary level (cystitis, erectile dysfunction, vaginal dryness and stenosis, infertility and teratogenicity), but also at psychological level with fatigue and depression (for review [51]).

Proton therapy studies reported approximately the same proportion of early toxicities than photon therapy. However, comparative studies to photons are still necessary when possible. Recent reports tend to show a decrease in early and late toxicity: Romessser et al. [52] reported that proton therapy for head and neck cancers had significantly lower rates of early grade 2 (grade represents the degree of gravity of toxicity) or greater acute dysgeusia (5.6 vs. 65.2%), mucositis (16.7 vs. 52.2%) and nausea (11.1 vs. 56.5%). Yock et al. [53] reported ototoxicity and neuroendocrine deficit, but no cardiac, pulmonary or gastrointestinal late effects after treatment of medulloblastomas by protons, with a median follow-up of 7 years.

First studies of patients undergoing carbon ion therapy and presenting side effects were reported during the end of the 2000s (for review, [26]). Comparative studies on toxicities

of carbon ion therapy versus conventional radiotherapy are still missing. Concerning bone and soft tissue sarcomas, toxicities were mostly decreased compared to conventional radiotherapy. A report showed that, on 78 patients treated by carbon ion therapy for unresectable osteosarcomas, grade 3 acute and late skin reactions were seen in 3 and 4 patients, respectively, and grade 4 skin and soft tissue reaction occurred in 3 patients [54]. However, for an escalation dose protocol, toxicities were considerably increased: 34 on 35 patients present acute skin reactions and 26 on 27 patients late skin reactions up to grade 4 [55]. For unresectable sarcomas: on 47 patients treated for non-sacral spinal sarcomas, 1 patient presented grades 3 and 4 late skin reaction and 1 patient grade 3 spinal cord reaction [56]; 6 patients and 2 patients on 188 patients with sacral chordomas presented grade 3 peripheral nerve and grade 4 skin toxicity, respectively [57]; and 4 patients on 75 patients treated for non-skull base chondrosarcomas report grade 3 or 4 late skin and soft tissue reactions [58]. Except bone and soft tissue sarcomas, most of toxicity was encountered for cervical cancers: a dose escalation protocol led to 18% of major gastrointestinal toxicity [59], and in another study, 8 patients on 29 developed bladder complications and 4 patients presented grade 4 rectal toxicities [60]. Clinical trials are in progress to register toxicities in the different facilities providing carbon ion therapy [26].

Induction of secondary tumors was also reported. Concerning protons, Chung et al. [61] studied 558 patients treated by protons and 558 treated by photons: second malignancies occurred in 5.2% of proton patients compared to 7.5% of photons. They concluded that proton therapy was not associated with a significantly increased risk of secondary malignancies compared with photon therapy, but the follow-up of these patients was only around 6 years after radiation therapy. This reduced risk of secondary malignancies due to proton therapy was confirmed by Sethi et al. [62], whereas there are no enough long-term reports after carbon therapy. Indeed, concerning carbon ions, literature on secondary tumors is still poor but a study pointed out that 30% of patients treated for cervical cancers developed distant metastases [63]; a case was reported of a brain tumor induced by heavy particle radiotherapy [64]. Preclinical studies, recently performed on mice exposed to carbon ions in comparison to photons, revealed that interstitial chromosome deletions were more increased in secondary cancers induced by carbon exposure [65]. They contradict previous results of Ando et al. [66] showing the same induction in carbon locally irradiated mice of secondary tumors after γ-rays.

3.2. DNA damage and repair, mitotic catastrophe

Production of clusters of DNA damage can lead to mitotic catastrophe in fast or slow renewal normal tissues then leading to early or late toxicities. A lower immediate increase in DNA damage measured by alkaline comet assay was observed in confluent primary cultures of skin fibroblasts after carbon ion versus X-ray irradiation but a late increase in DNA damage was observed only after carbon ions whereas it was not the case after X-rays [3]. The lower immediate increase could be explained by the production of smaller fragments after carbon ions compared to X-rays whereas the late production of DNA damage after carbon ions could come from DNA repair. Indeed, micronucleus frequency—described as a result of impaired repair of DNA double-strand breaks—was 1.7-fold increased 24 hours after carbon irradiation compared to X-rays (unpublished results) and this increase persisted 2 weeks after irradiation (unpublished results) where a late wave of oxidative damage was observed [3].

Results obtained by Antonelli et al. [67] on quantification of γ-H2AX foci after carbon ion vs. X-ray irradiation in lung fibroblasts showed a longer persistence of γ-H2AX foci after carbon ion irradiation which is in agreement with a more difficult repair of DNA complex damage. Moreover, Gustafsson et al. [68] studied, in normal human skin fibroblasts, clustered DSB and non-DSB lesions which convert into DSB during preparation for pulsed-field gel electrophoresis and their results showed a similar increase after carbon ion or low-LET irradiation. It was recently shown that clustered DSB perturb normal human fibroblast DNA repair after high LET irradiation [69]. In confluent normal fibroblasts, accumulations of p53 at early times and p21 at late times were 2–3 times higher after carbon ions than after X-rays [70]. DNA repair proteins (hMRE11, p21, PCNA) were accumulated along ion trajectory in normal human fibroblasts and this was dependent of chromatin compaction [71].

3.3. Role of oxidative stress in hadrontherapy toxicity

Highest toxicity of carbon ions, and in a lower extent of protons, could come from indirect effects of irradiation, i.e. due to a stronger concentration of reactive oxygen and nitrogen species that cells would not be able to detoxify. However, only few studies were interested in oxidative phenomena occurring after carbon ion or proton irradiation.

Wan et al. [72] showed that ROS production in human epithelial cells occurred in the same proportion after proton or X-ray irradiation. Whole body proton irradiation of mice also led to an early differential modulation of oxidative stress gene expression in liver: only proton irradiation led to an increase in Prdx6 and Sod3, mainly, whereas other genes were common to photon irradiation [73]. Chang et al. [74] demonstrated that whole body proton irradiation of C57BL/6 J mice leads to a late increase in ROS production, NOX4 transcription and DNA damage in hematopoietic stem cells from irradiated mice. Proton irradiation of rat eye led to an upregulation of oxidative stress and apoptosis gene expression [75]. Baluchamy et al. [76] concluded that, after proton irradiation, mouse brain presented modifications in expression of genes related to oxidative stress which could lead to programmed cell death. Moreover, the use of antioxidants allowed to protect against biological effects of protons not only *in vitro* [77] but also *in vivo* [78], which tends to demonstrate the importance of oxidative stress. Transgenic mice overexpressing human mitochondrial catalase presented protective effects on low-dose proton-induced brain injury [79]. In the same manner, neuroprotective effects of reducing mitochondrial ROS were also shown by Liao et al. [80] in proton irradiated mice not only at low dose but also at a higher dose of 2 Gy. SOD mimetic was also shown efficient in reducing oxidative damage in retinal cells from proton eye-irradiated rats [81] and in ameliorating acute and chronic proctitis in focal proton irradiated rat rectum [82].

After carbon ion irradiation, an increase in oxidative stress was observed in confluent irradiated primary cultures of normal human skin fibroblasts with an increase in biological macromolecule damage and a decrease in antioxidant enzyme activities in comparison with X-rays [3, 83]. This trend was confirmed by Dettmering et al. [84]: an increase in superoxide anion production was measured in normal human fibroblasts and the maximum level was obtained at a lower dose after carbon irradiation than after X-rays. In human hematopoietic stem/progenitor cells (HSPCs), carbon irradiation led to a strong increase in heme oxygenase-1

and NAD(P)H dehydrogenase-quinone 1 expression [85]. *In vivo*, mouse whole-body carbon irradiation was shown to decrease glutathione level and to increase MDA content in testis one week after irradiation [86]. At longer term - 2 months after exposure - and in comparison to gamma-rays, mouse whole body irradiation led, in intestine and colon, to: (i) a persistent increase in ROS, mitochondrial cardiolipin oxidation and lipid damage; (ii) a late decrease in antioxidant enzyme activities [87]. The use of other antioxidants indirectly pointed out an important role of oxidative phenomena. Indeed, some antioxidants allowed to decrease effects of carbon ions in normal cells or tissues: curcumin ameliorates cognitive deficits in carbon-irradiated mice via SOD increase, MDA decrease and upregulation of important genes in oxidative stress pathways like heme oxygenase-1 and NAD(P)H quinine oxidoreductase 1 [88]; melatonin reduced carbon-induced apoptosis in mouse carbon-irradiated testes [89] and brain [90] via a decrease in carbonyl and MDA content and an increase in SOD and catalase activities; Dragon's blood decreased hydrogen peroxide and MDA levels and increased SOD activity and glutathione content in carbon-irradiated rat brain [91]. These last experiments provide indirect proofs of the major role of oxidative stress in hadrontherapy toxicity.

3.4. Stress-induced premature senescence

In normal human fibroblasts, radiation exposure lead to a G1 cell cycle arrest evolving in quiescence or senescence [92]. Premature senescence, or SIPS (stress-induced premature senescence), differs from replicative senescence. SIPS phenomenon was generally observed in fibroblasts exposed to prolonged or repeated stresses [93, 94] and was also shown after X-ray exposure [95, 96]. Naka et al. [96] showed, by the use of ATM mutated fibroblasts, that pathway leading to premature senescence in fibroblasts after oxidative stress or X-ray exposure could also be ATM-dependent and could act via p38MAPK and p16INK4A. After carbon ion exposure, a higher accumulation of p21 in carbon-irradiated confluent normal fibroblasts was observed at late times compared to X-rays [70]. In normal human lung fibroblasts, carbon ion irradiation led to a faster senescence than γ-rays [97] However, this phenomenon of premature senescence was observed in the same proportion as for X-rays in the progeny of human fibroblasts after an immediate cell cycle arrest and senescence reappeared and persisted after 5 months after exposure [98]. Our experiments on confluent primary cultures of normal human skin fibroblasts showed a lower proportion of senescence-associated β-galactosidase cells 3 weeks after carbon ion exposure compared to X-rays (unpublished results) (**Figure 3**).

3.5. Inflammation and late toxicity

Schematically, acute side effects in normal tissues would be generally related to a loss of fast renewal cells, whereas late effects would appear due to several more complex phenomena as the loss of low renewal cells, progressive ischemia due to the loss of microvascularization endothelial cells and the development of late fibrosis, mainly due to inflammatory processes [99, 100]. After irradiation, it is known that cytokines, which are important mediators of late radiation-induced effects, are not only secreted at early times after irradiation but also at later times-months or years after exposure. Normal tissues monocytes and macrophages produce proinflammatory cytokines like IL-1, IL-6 and TNF-α, which attract macrophages and
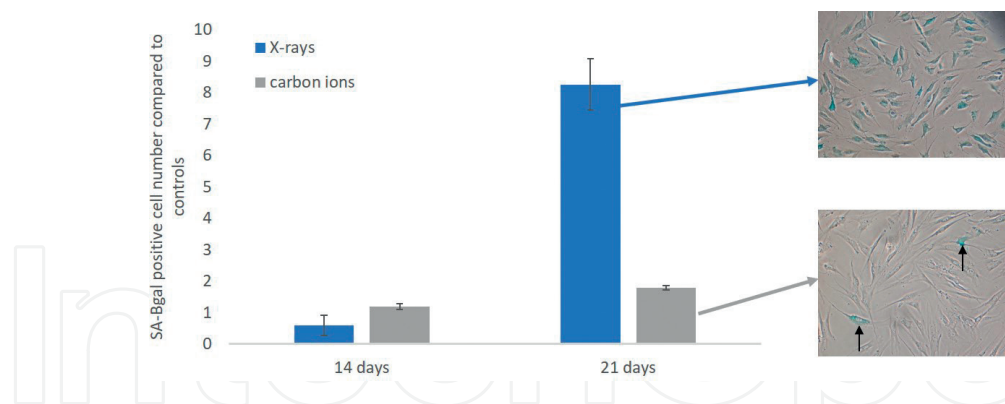
Figure 3. Premature senescence in normal human skin fibroblasts exposed to carbon ions or X-rays at an isosurvival dose (unpublished results). Cells were irradiated at confluence and kept until 14 or 21 days post irradiation. After fixation, SA-β-galactosidase staining was performed (citric acid/sodium phosphate 40 mM, NaCl 150 mM, MgCl$_2$ 2 mM, potassium ferrocyanide 5 mM, potassium ferricyanide 5 mM and X-gal 1 mg/mL). SA-β-galactosidase activity was determined by counting blue cells using a microscope. Data represent mean percentage of β-gal positive cells ± SEM.

lymphocytes. Activated macrophages and stimulated stromal cells synthetize fibrogenic cytokines such as TGF-β and PDGF modulating fibroblast proliferation-differentiation balance and protein synthesis and degradation via metalloproteinases (MMP) and their inhibitors (TIMP) (for review, [99, 101]). In this way, specificity of proton or carbon ion irradiation concerning these pathways is of main interest to modulate late effects of hadrontherapy. Fournier et al. [102] showed an accumulation of fibrocytes and extracellular matrix proteins in normal human foreskin fibroblasts exposed to carbon ions. However, a lowered increase in IL-6 was observed in normal human skin fibroblasts exposed to carbon ion compared to X-ray irradiation [3]. The use of Dragon's blood, which presents antioxidant and anti-inflammatory properties, did not allow to reduce TNF-α, IFN-γ and IL-6 levels in carbon-irradiated rat brain as it was the case for γ-rays [91]. A recent report on carbon-irradiated normal human skin models showed similar inflammatory processes than after the same dose of X-rays [103].

3.6. Bystander effects

Bystander effect, i.e. biological effects to cells which were not irradiated via signals coming from irradiated cells, could be at the origin of normal surrounding tissue injury and to, for example, abscopal effects. Oxidative stress signal pathways could play an important role in these effects.

Indeed, confluent human skin fibroblasts were shown to present a persistent oxidative stress after exposure of 0.036–0.4% of them to proton or X-ray microbeam but this was not the case for carbon ions [104]. However, when normal cell cultures exposed to low-LET protons were co-cultured with unirradiated cells and after 20 population doublings, no changes in survival, chromosomal damage, protein oxidation and lipid peroxidation were observed [105]. This was not the case for higher LET (iron and silicon ions) for which a higher level of oxidative damage, a decrease in antioxidant enzyme activities and an alteration of mitochondiral proteins - encoded by mitochondiral DNA - were observed [105].

Recently, Autsavapromporn et al. [106] have shown that glioblastoma cell carbon irradiation led to damage in unirradiated normal fibroblasts. Moreover, a single dose of carbon irradiation

Time after irradiation	8-oxodG concentration (nM)	
	24 hours	14 days
X-ray control	0.775 +/- 0.140	0.817 +/- 0.036
X-ray irradiated	1.980 +/- 0.064	2.175 +/- 0.073
C-ion control	0.822 +/- 0.103	0.830 +/- 0.084
C-ion irradiated	3.103 +/- 0.296	3.153 +/- 0.262

Table 1. 8-oxodG concentration in normal human skin fibroblast culture supernatants exposed to carbon ions or X-rays at an isosurvival dose (unpublished results).

led to less damage than a fractionated dose. However, after 20 population doublings, there were more damage on cells irradiated in one time than in several fractions. Dose hypofractionation, which is presented as a major advantage of carbon therapy, could therefore engender more late effects to bystander normal tissues. Inflammatory pathways playing an important role in oxidative stress persistence in normal tissues after irradiation thus leading to normal tissue injury, the study of bystander effects on the secretion of inflammatory cytokines is of major interest. Carbon microbeam irradiation of a low proportion (0.45%) of immune cells led to decreased cytokine levels [107]. Oxidized extracellular DNA could also be a signaling factor in bystander effects. 8-oxodG is the main oxidatively generated DNA lesion and is formed either by direct oxidation or can be incorporated in DNA from oxidized nucleotide pool by DNA polymerase. Its extracellular presence can be due to DNA repair, cell death, mitochondrial turnover, cellular uptake or salvage of DNA damage products. Carbon-irradiated confluent skin fibroblasts exhibited a 1.5-fold increase in extracellular 8-oxodG 24 hours and 2 weeks after C-ion beam exposure compared to X-rays (see **Table 1**, personal unpublished data). In this way, the role of bystander effects in carbon or proton therapy remains unclear and needs further investigations.

Cell culture supernatants were purified by solid phase extraction, and samples were adjusted for the standard addition method in order to correct for the matrix effects contributed by the culture medium constituents as reported previously [108]. An optimized method for the quantification of 8-oxodG has been applied. HPLC-ECD signals were recorded in the culture supernatants spiked with the external standard. Data represent mean 8-oxodG concentration ± SEM.

4. Conclusion

Mainly due to the cost of hadrontherapy facilities, there are too few studies dealing with biological effects of protons, carbon ions or other particles on tumors and normal tissues. In addition, a large proportion of these works did not compare carbon ion effects to X-ray effects. Advantages of hadrons, mostly on tumors, are often highlighted but particular attention should be paid on side effects of hadrons, especially hypofractionation which could lead to major injuries in normal tissues. Killing efficiency of carbon ions is often considered lower for normal cells than for tumor cells. However, some recent studies tend to show a strong increase in oxidative stress in normal cells after protons [74, 79] or carbon ions [3, 84].
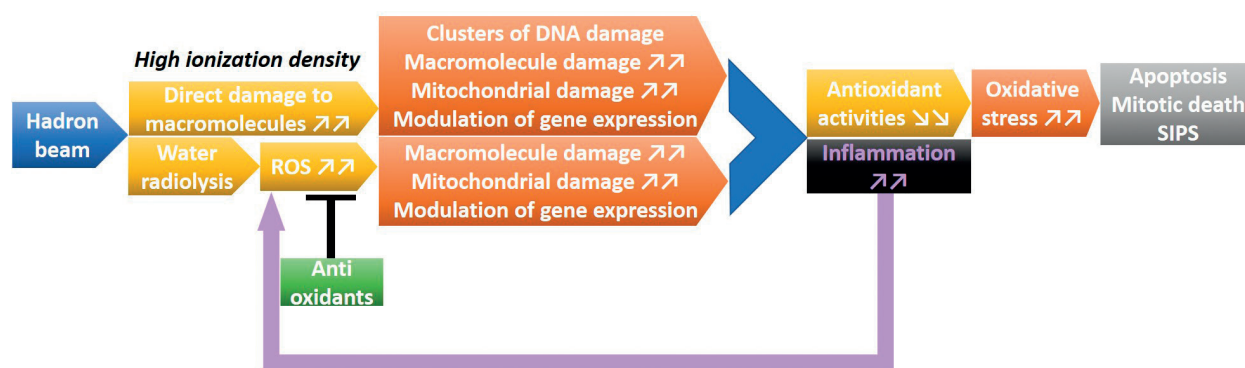
Figure 4. Proposed schema of biological effects of hadron irradiation leading to tumor killing and normal tissue toxicity.

According to the literature, **Figure 4** proposed a schema of biological effects leading to tumor cell death or to normal cell toxicity.

Further investigations are needed to better understand toxicity of protons and carbon ions. Prediction of side effects for each patient should be of major interest in order to adapt radiotherapy protocol and/or to prevent deleterious effects due to normal tissue irradiation or to bystander phenomena. The use of antioxidants, which were demonstrated as efficient in reducing late effects of protons and carbon ions, could be of major interest in preserving normal tissue during proton or carbon ion therapy. Another guideline for reflection is related to the drawbacks of protons and carbon ions: they could lead to an interest of other ions as helium ions which should lead to less toxicity in normal tissues but are also less efficient on cancer cells and which do not present the same interest as carbon ions in killing tumor cells in hypoxic conditions. In conclusion, due to complex effects of hadrons when encountering normal tissues and tumors, there is a strong need in preclinical studies—at early and late times post-irradiation and in comparison to photons—to determine biological effects of SOBP, ion fragmentation, LET distribution in depth, hypofractionation, beam scanning, etc.

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