Egypt. J. Biophys. Biomed. Eng., **Vol. 26,** pp: 33-43 (2025)

Egyptian Journal of Biophysics and Biomedical Engineering
https://ejbbe.journals.ekb.eg/

Pulsed Low Dose Rate Radiation Therapy Effects on Hepatic Tumor Rat Model

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THE aim of this study is to examine the effectiveness of pulsed low-dose-rate radiation therapy (PLDR) in controlling hepatic cancer. Hepatocarcinogenesis was induced in rats using diethylnitrosamine (DENA). The rats were classified into 4 groups, negative control group, positive control group, conventional radiation group (CRT) and PLDR group. Both irradiated groups received 6Gy to the liver using 6MV beams. Body weight was observed daily. Tumor volume was measured using computed tomography scan (CT). Liver tissues were subjected to histopathology. Alfa fetoprotein (AFP) and interleukin 6 (IL-6) level were assessed. Both CRT and PLDR techniques could significantly inhibit hepatic tumour growth when compared with non–irradiated group (P value < 0.05), PLDR technique showed better results compared to the CRT technique, but the difference was not significant, P >0.05. PLDR group showed a continuous increase in weight, while CRT group showed gradually weight loss until the 5th day. Both groups demonstrated a significant decrease in AFP and IL-6 serum level compared to the positive group. The estimated AFP and IL-6 in PLDR were lower than that of CRT (P=0.16 and P=0.001, respectively). The average levels of serum AFP were 0.554 ±0.11, 2.52± 0.23 ,1.258± 0.39 and 0.976± 0.35 and for IL-6 were 16.01± 2.5 ,46.9±2.9, 27.5±1.5 and 22.5±1.03 for negative control, positive control, CRT and PLDR, respectively. Histological examination indicated more liver cell degeneration with CRT. Hepatic tumors could be controlled with PLDR at least as successfully as CRT, with a beneficial advantage of showing significantly less normal tissue toxicity.

Key words: PLDR, CRT, AFP, IL-6, hepatic cancer.

Introduction

Radiation therapy (RT) has been developed overtime as a viable treatment option for hepatic cancer. It is usually recommended as an effective treatment for most patients with inoperable and/or locally advanced hepatocellular carcinoma (HCC)[1]. The technical advancements in the radiotherapy machines and equipment promoted an increase in its application in treating hepatic tumors [2]. Modern radiotherapy techniques like stereotactic body radiation therapy (SBRT) have enabled high focal doses of radiation to be delivered to patients with different liver malignancies [3]. SBRT is often preferred when tumor distribution, size and/or multi-focality are out of range of surgical resection. However, not all patients with liver tumors can be treated with SBRT because of the risk of exceeding the radiation tolerance of normal liver [4]. Radiation-induced liver disease (RILD) is one of the most devastating consequences of radiation to the liver [3]. The frequent association of RT with concurrent liver cirrhosis is a major challenge in radiotherapy [2]. Currently, no pharmacological therapy can relieve RILD symptoms. Several studies have been directed toward decreasing toxicity. For example, Wu et al conducted a study to define the dose that results in RILD. Based on their study, toxicity increased and RILD developed with a dose $\geq 31.76 \pm 1.94$ Gy[1]. Li et al studied the effect of using curcumin concurrent with liver irradiation in rats, they highlighted that curcumin treatment reduces the liver damage and toxicity caused by irradiation[5]. Alkhalf and Khalifa tried to reduce the harmful effects resulting from liver irradiation using blueberry extract. This extract contains antioxidant compounds such as anthocyanins and phenolic acids. They found that using them as a diet supplement can improve antioxidant defense systems and immune-system parameters in rats exposed to irradiation [6]. Sundram and Buscombe investigated the use of selective internal radiation therapy (SIRT) for treatment of liver tumors. The SIRT technique relies on the administration of a radioactive substance into the tumor vascular supply via an intra-arterial catheter placed under radiological guidance. The aim is to deliver a

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Received: 7/12/2024; Accepted: 14/12/2024
DOI: 10.21608/EJBBE.2024.342628.1080

tumoricidal dose of radiation (>100 Gy) to tumor tissues, with relative sparing of adjacent normal liver parenchyma [7]. Petersen et al investigated the potential sparing of normal liver by intensity-modulated proton therapy (IMPT) compared to photon-based intensity-modulated radiotherapy (IMRT) for solitary liver tumors [4]. Despite those mentioned studies, we believe more research efforts are needed to find better alternative techniques. Pulsed low-dose-rate radiotherapy (PLDR) is a new delivery radiation method that has been used clinically for re-irradiation in different recurrence cancer cases. PLDR has been shown to result in higher normal tissue radiation tolerance [8,9]. PLDR treatment is designed to give pulses of radiation below a threshold dose that is usually called the transition dose. A dose higher than the transition dose will change the cancerous cells from a sensitive (hyper radio sensitivity) response to resistance response. It was shown that many cancerous cell lines can have hypersensitivity with low doses of radiation and on the other hand the low dose rate can result in higher normal cells repair. [10 -13] This is cell type dependent and has been observed in the dose range 0.2Gy-0.6 Gy[11, 14-17]. The transition dose of tumor cells are greater than that of normal cells[18,19]. Consequently, normal tissues repair is triggered during PLDR while less repair will be seen with tumor cells. PLDR is usually applied by delivering the treatment fraction 2Gy in a series of 0.2 Gy pulses separated by 3 minutes intervals, resulting in an effective dose rate in the order of 0.0667 Gy/min [8]. Although, the dose rate effect has been studied, the damage repair mechanism is still not obviously clear [12, 20-25]. The dose rate effect in DNA repair can be observed in the range between 0.01Gy/min-1Gy/min[20,26]. A number of studies have been conducted to unveil the mechanism of PLDR in tumor controlling of different tumor sites versus the conventional radiation delivery [26-33]. These published research data allowed useful information to support other trials for various tumor sites. Thus, in this study we will study the use of PLDR for treatment of liver lesions. The aim is to reduce the risk of RILD as it remains a challenge that limit the usage of external beam radiation therapy (EBRT) in the treatment of malignant hepatic tumors [34]. As far as our knowledge, up till now no previous research on hepatic cancer has used PLDR. We implanted a hepatic cancer model in rats to investigate if we could achieve any potential gain in therapy and a reduction in toxicity for hepatic cancer treatment with PLDR.

Material and methods

Animal tumor model

Adult Wistar pathogen -free male rats, approximately 8 weeks old, weighing 130 ~ 150g were obtained from faculty of Medicine Ain Shams Medical Research Institute (MASRI). They were housed in polypropylene cages under standard laboratory conditions (temperature 25^0C $\pm 2^0$C, 50 ± 10 % relative humidity and 12 hours dark/light cycle). The rats were fed with a standard diet and drinking water ad Libitum. All the animals were acclimatized to the environmental conditions for a week before starting the study. Hepatocarcinogenesis was inducted by diethylnitrosamine (DEN) which was purchased from Sigma Aldrich Chemical Co., USA. The animals received orally a dose of DEN equal to 20 mg/kg body weight five times per week within 8 weeks (equivalent to 800 mg/kg of rat).[35,36]. Then liver tissues were harvested and fixed in phosphate-buffered 10% formaldehyde for histopathological examinations to check the occurrence of hepatocarcinogenesis.

Experimental Design

The animals were divided into four groups. The negative control group or normal rats (n=12) received only 0.9% normal saline daily through the period of tumor induction. The other three groups were tumor bearing rats. A positive control group did not receive any radiation treatment (n=12). PLDR group (n=12) received 6 Gy delivered in thirty 0.2 Gy pulses with 3 minutes interval break. CRT group (n=12) received 6 Gy with conventional dose rate.

Ct imaging

The hepatic tumor was imaged using Toshiba scanner Aquilion (TSX-201A). Images were taken using 80kV tube voltage, 100mA, and 230mm field of view. The CT scans were done using 2 mm slice thickness. Rats were anesthetized during the CT scan with an intramuscular injection (I.M) of a Ketamine Alfasan 10% (Alfasan Nederland B.V., Kuipersweg 9, 3449 JA Woerden, The Netherlands) with dose equal to 15mg per kg body weight (0.15 ml/kg body weight). Lead marks and tattoo ink dots are applied to the rat's skin to identify the area for treatment. These marks will also be used for rats positioning during treatments.

Irradiation

The radiation treatment for the hepatocarcinoma in the PLDR and CRT groups were delivered using 6 MV photon beam from our Unique linear accelerator (Varian Medical System, USA, PA). The dose rate used for each PLDR pulse was 100MU/min separated by 3min beam off interval breaks to achieve the 0.067 Gy/h effective dose rate. The dose rate used for CRT was 400MU/min. IM ketamine and ethanol were used to anaesthetize the rats, then rats were positioned head-first prone on the treatment couch. A thermoplastic mask that outlined the rat body and lead marks were used to ensure that the rats are positioned similar to their position

during their CT scan. Treatment was performed using an open beam with a field size of 4 x 3 cm^2 and 671±15 MUs to deliver 6 Gy dose to the liver.

Tumor Volume Measurement

The liver volume was monitored using CT scans taken on the day of treatment prior to irradiation and with CT scans taken weekly after irradiation. Liver contouring was performed using Eclipse treatment planning system (Varian, USA, V.13.5). Liver size was measured for depicting tumor volume. Tumor volumes of each individual rat at different time intervals were normalized to their initial volume prior to treatment to calculate the relative volume change. Rats' changes in body weight after irradiation were monitored daily during the experiment.

Histopathology

The liver tissues were harvested from rats, placed into cassettes, and submerged in 10% neutral buffered formalin to be embedded in paraffin blocks for histopathological examination. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degrees in hot air oven for twenty-four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by Hematoxylin and Eosin (H&E) stain for examination by LABOMED Fluorescence microscope LX400, cat no: 9126000; USA.

Blood sampling and biochemical analysis

The rats were sacrificed following 2 weeks of irradiation. Blood samples were collected, serum was separated for the estimation of alpha-fetoprotein (AFP) and Interleukin 6 (IL-6) using a rat alpha-fetoprotein ELISA kit (E-EL-R0153) and a rat IL-6(Interleukin 6) ELISA Kit (EL-R0015). The kits were purchased from Elabscience Biotechnology Co., Ltd. The tests were done according to the manufacturer's instructions.[37,38].

Statistical analysis

The data were analyzed statistically using Microsoft excel (Microsoft 365). The mean and standard deviation of the mean (SDM) (standard error) were calculated, and the results were represented as mean ± SDM using error bars. Student's t-test with one tail to determine any significant difference between the groups with a p-value threshold ≤ 0.05. The standard errors of the mean, which were calculated using the equation of standard deviation divided by the sample number.

Results

Weight observation

The Rats in CRT delivery group exhibited lower food intake and activity levels. They showed a significant reduction in weight compared to PLDR following irradiation until the 5th day with p-value= 0.003.Then, they started to increase in weight from sixth day as shown in figure 1. On the other hand, PLDR group didn't show any weight loss, they showed a gradually increase in weight from 1st day after irradiation. A significant difference is shown between the 2 groups (P value=0.0009).

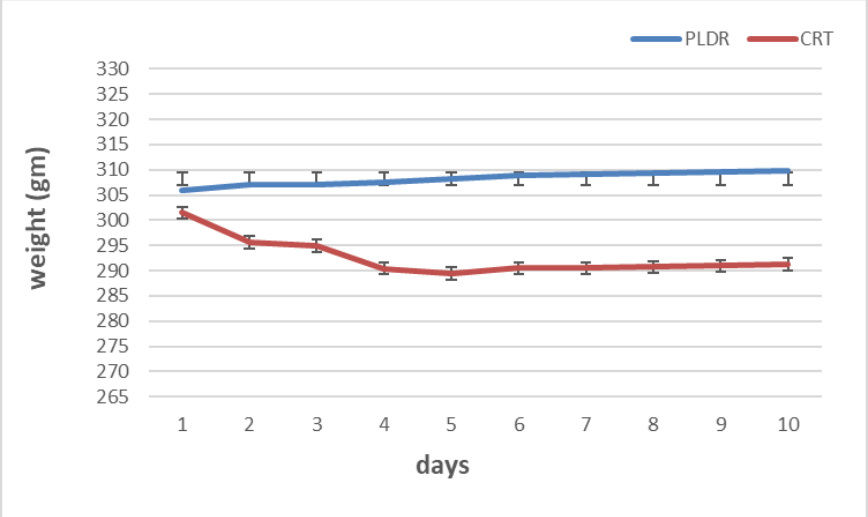

Fig. 1. Observations of daily rat's weight in CRT group treated with 6 Gy using conventional dose rate compared to the group treated with PLDR (30 pulse * 0.2Gy with beam off =3 minutes).

Histopathological examination of liver tissues

The histological analysis of the liver sections from the negative control group stained with H&E revealed a normal histoarchitecture of the liver. It showed a typical histological pattern of hepatic cells with normal hepatic nodules and central vein (Figure2). Liver sections of positive control group showed a moderately large area of vascular congestion and extravasation. Focal lymphoplasmacytic infiltrates were seen at periportal and perivascular as well as in intra-acinar areas. Hepatocytes show diffuse ballooning degeneration associated with regenerative cellular changes. In addition, grade II fibrosis was detected mainly around the biliary ducts and perivascular spaces as well as widening in the intralobular spaces (Figure 2).

Two weeks after irradiation, CRT liver specimens showed a small localized vascular congestion and extravasation. Intra-acinar infiltration of lymphocytes, which is associated with regenerative hepatocytes as indicated by moderate enlarged cells, low nuclear /cytoplasmic ratio, and ballooning. In addition, in acidophilic bodies and binucleated hepatocytes are detected (Figure 3). Liver tissues from PLDR group after 2 weeks of irradiation exhibit a few lymphoplasmacytic infiltrates were seen at periportal and perivascular areas. The hepatocytes show regenerative cellular changes in the form of nuclear enlargement, acidophilic bodies, and binucleated hepatocytes (Figure 3).

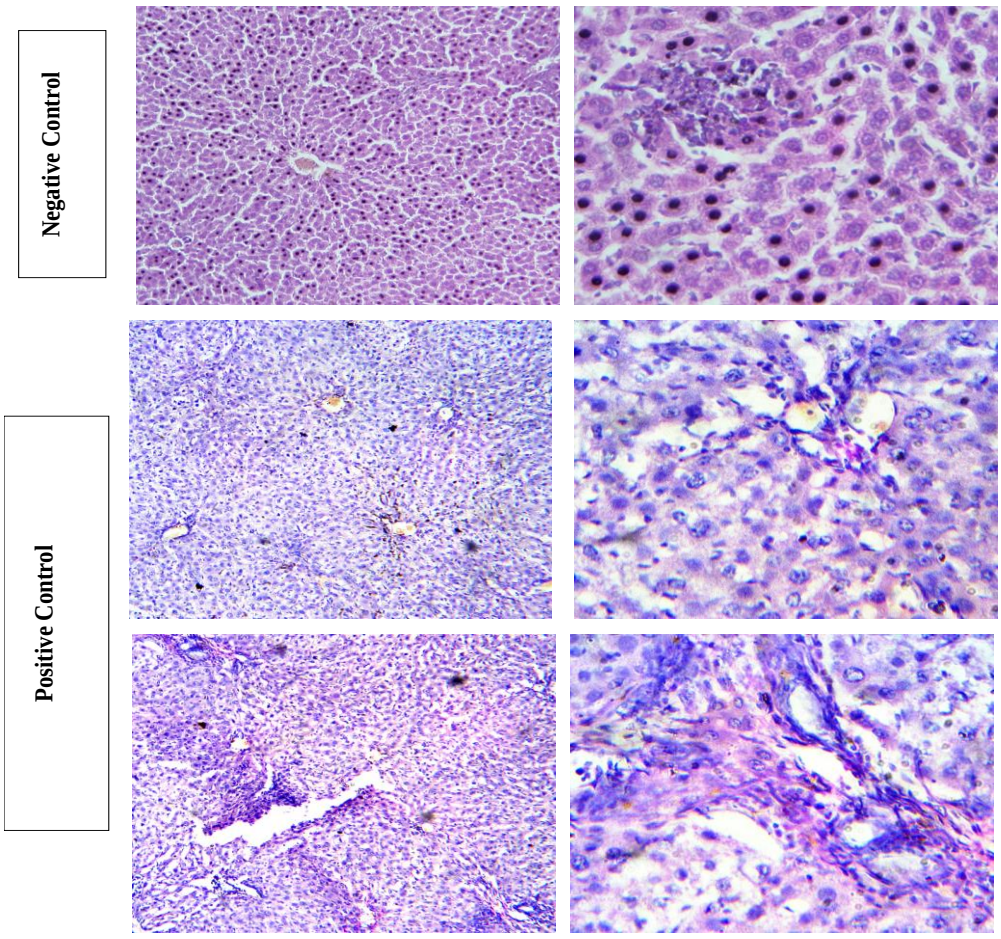

Fig. 2. Sections from Liver specimen from positive control rat group. The images magnifications are X10 (left side) and X40 (right side). The images were captured with LABOMED Fluorescence microscope LX400, cat no: 9126000; USA.

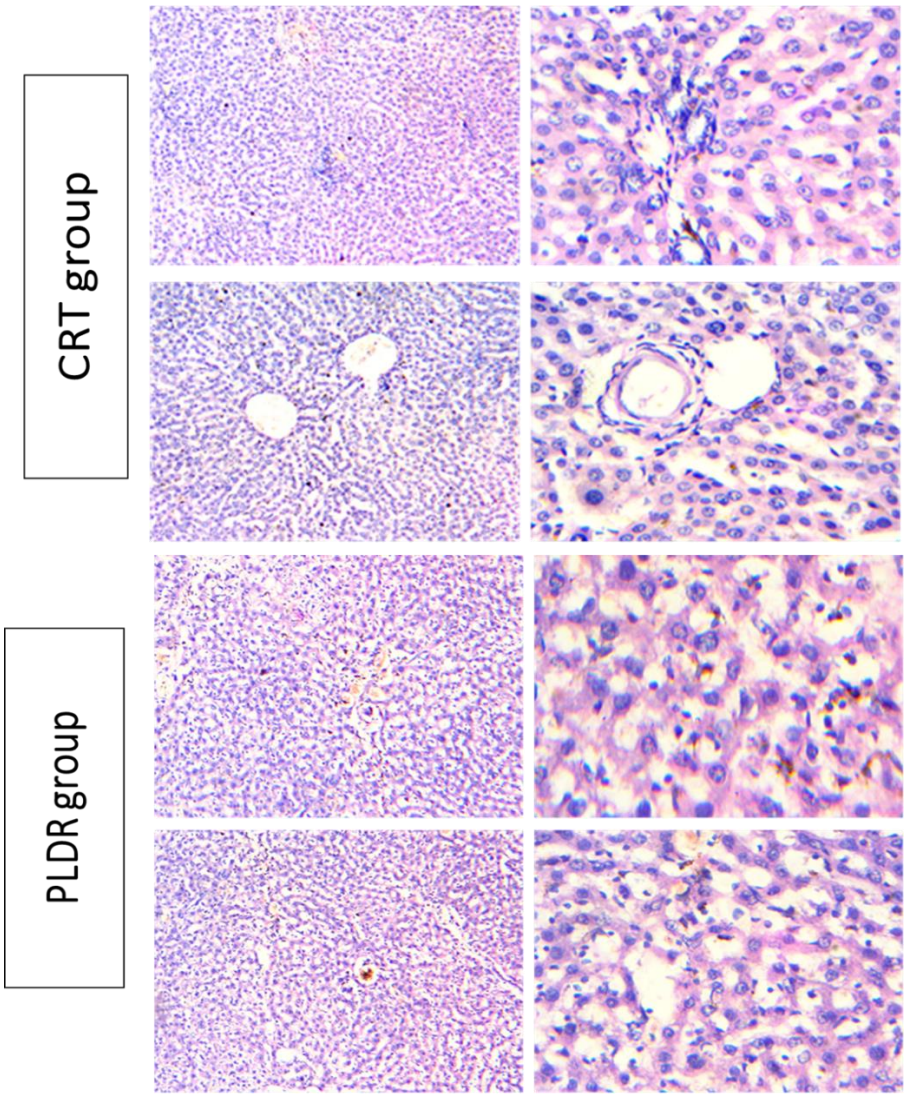

Fig. 3. Liver specimen from rats of CRT group and PLDR group. Stained with Haematoxylin and Eosin (H & E) then examined microscopically with light microscope. The images magnifications are X10 (left side) and X40 (right side). The images are captured with LABOMED Fluorescence microscope LX400, cat no: 9126000; USA.

Tumour growth analysis

The hepatic masses were scanned in the day of the treatment (0W) and then weekly for 2 weeks after irradiation with 6 Gy for determining the hepatic growth curves in the rats (Figure 4). The mean tumour sizes on the day of the treatment (0W) were 6.066±1.17mm^{3,} 6.03±1.06mm^3 and 6.05±0.77mm^3 for positive control group, CRT group and PLDR group, respectively. Then the mean size one week following irradiation (1W) were 6.5±1.13mm^3,5.51±0.91 mm^3 and 4.76±1.08mm^3, and on second week of the irradiation (2W) were 7.38±1.22 mm^3, 4.7±0.9 mm^3 and 4.2±1.11 mm^3 for positive control group, CRT group and PLDR group, respectively. The growth of hepatic mass of PLDR and CRT groups was compared with the tumour growth of non-irradiated group (positive control group). The results showed that both CRT and PLDR techniques could significantly inhibit hepatic tumour growth when compared with non–irradiated group (P value < 0.05). PLDR technique was showing better control than CRT technique, but the difference was not statistically significant with a P value = 0.22 for 1st week and 0.42 for 2nd week. (Figure 4) This indicates that PLDR will be at least comparable to CRT in delaying the tumour growth.

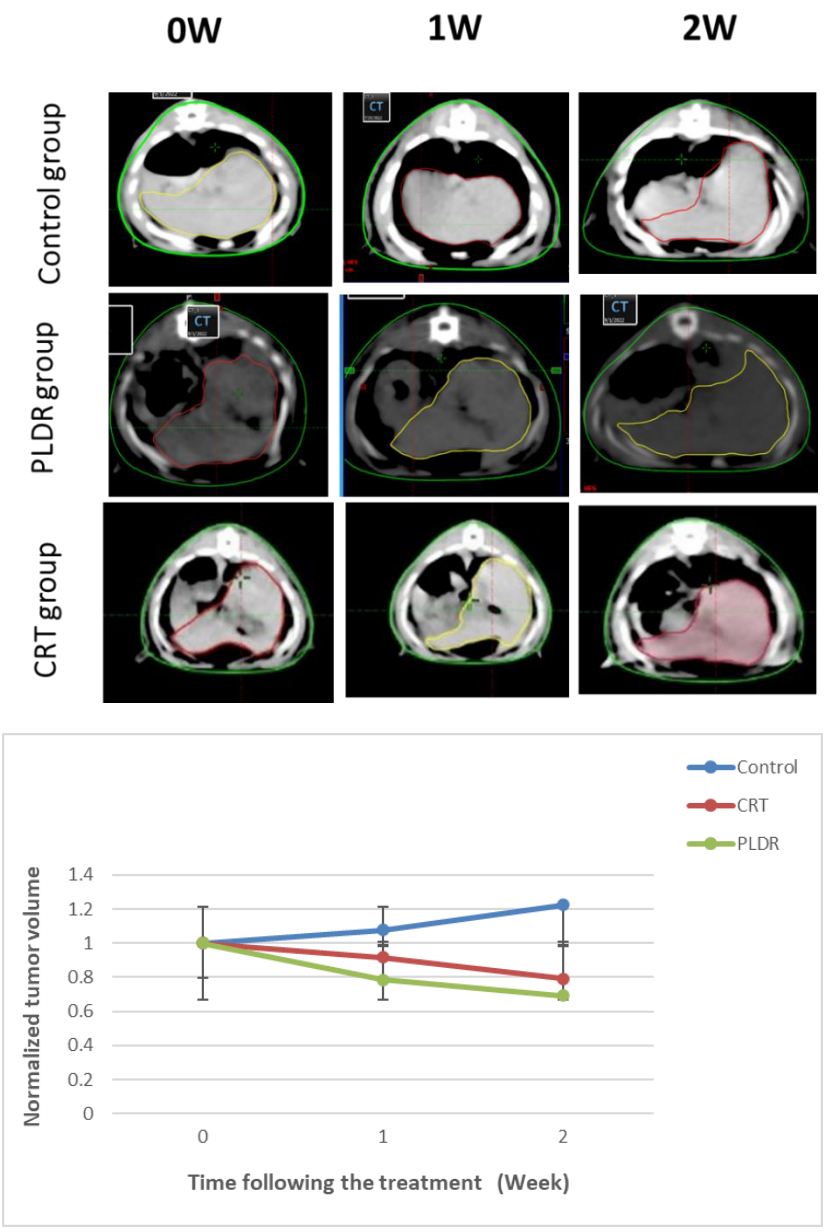

Fig. 4. Upper image is Ct images for Control, PLDR and CRT groups following treatment with 6Gy (W is week after treatment). Lower image is a plot of the normalized induced hepatic volume versus time in weeks of the three groups.

Biochemical analysis

There is a significant increase for AFP and IL-6 serum levels in positive control group in comparison to negative control group with P- value < 0.05. The average levels of serum AFP in the different groups were 0.554 ±0.11, 2.52± 0.23 ,1.258± 0.39 and 0.976± 0.35 for negative control, positive control, CRT and PLDR, respectively. The average levels of IL-6 were 16.01± 2.5 ,46.9±2.9, 27.5±1.5 and 22.5±1.03 for negative control, positive control, CRT and PLDR, respectively. (Figures 4&5). In both treated groups CRT and PLDR, there was a significant decrease in AFP and IL-6 serum level compared to the positive group (P < 0.05). Also, the results showed that AFP level in PLDR is lower than that of CRT, but the difference was not statistically significant having a P-value =0.16. On the other hand, IL-6 levels indicated that the inflammation was lower in PLDR and there was a high significant difference between the 2 groups (CRT & PLDR) having a P-value =0.001.

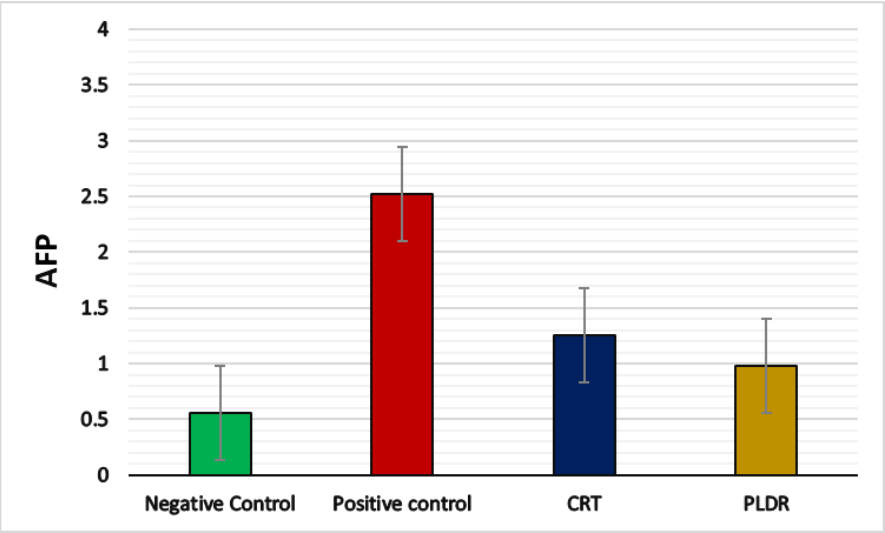

Fig. 5. AFP level of rat liver at 2 weeks after irradiation with 6Gy using CRT and PLDR. AFP (Alfa fetoprotein) ELISA Kit, Catalog No: E-EL-R0015, (*Elabscience Biotechnology, United states)*.

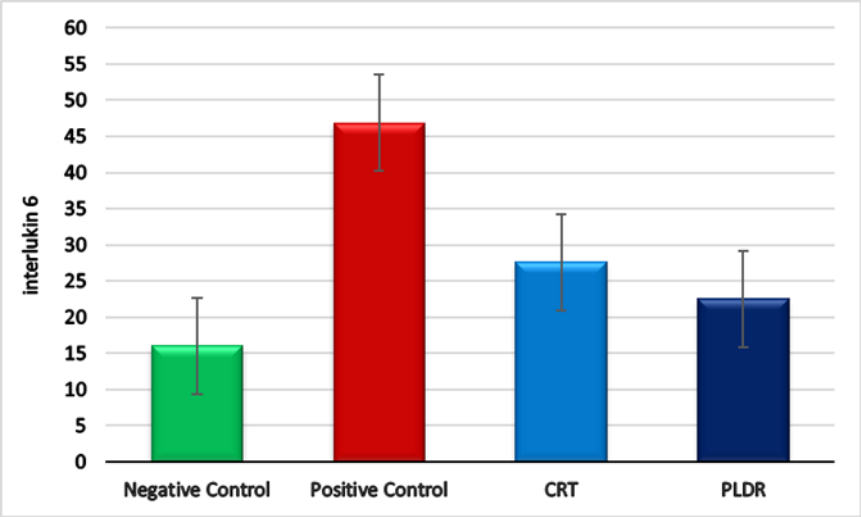

Fig. 6. IL-6 serum level of rat liver at 2 weeks after irradiation with 6Gy using CRT and PLDR. IL-6(Interleukin 6) ELISA Kit (EL-R0015), purchased from Elabscience Biotechnology Co., Ltd.

Discussion

RILD poses a chalenge in the treatment of liver tumors. It is even more complicated when a patient has a recurrent liver lesion with a prior radition to the liver. Reirradiation of recurrent tumors can result in a severe acute toxicity. There have not yet been a standard and efficious treatments available for recurrent malignancies or significant invasive malignancies of the abdomen [39,40]. It has been shown in in-vivo studies for different tumor sites like lung , prostate head &neck and GBM, that PLDR can increase normal cell resistance to radiation while maintaining a comparable tumor control as conventional delivery methods [29-33]. RILD can be either chronic or subacute [5,41]. RILD resulted in limitation for both the escalation of prescribed dose and reirradiation of recurrent cancers. [42] In this work, we investigated PLDR efficacy in an induced hepatic cancer model. Our data showed that PLDR can control liver tumors as effective as other conventional methods. This is in consistence with other studies done for othere treatment sites. For example, zhang etal assessed the controlling of A549 tumor lung model in mice using PLDR and wang et al evlauated the efficiacy of PLDR in controlling of 3 different prostate cancer models (orthotopic LNCaP tumor model, a flank PC-3 tumor model, and an orthotopic PC-3 tumor model). They both showed that PLDR could control tumors as comparable as conventional method with no statisical significant difference between both methods (p-value > 0.05).

The pro-inflammatory cytokine interleukin-6 (IL-6) has a critical role in the growth and advancement of the most prevalent kind of liver cancer, hepatocellular carcinoma (HCC)[43]. It is considered to be the most

predictor cytoine for HCC survival [44]. Numerous studies have revealed that IL-6 serum concentration was higher in hepatic cancer group than the normal control groups [44] .Furthermore, IL-6 belongs to the pluripotent cytokines which develope the inflamatory rersponse resulted from radiation treatment[45] and this multifunctional cytokine through some biological interactions could contribute in resistance to radiotherapy damage[46]. Any rise in proinflammatory cytokines, such as IL-6, indicates changes in inflammation[5]. Thus we used IL-6 to asses the difference between PLDR and CRT groups. The IL-6 serum quantitatively was signifacantly lower in PLDR than CRT, which indicate that PLDR delivery can reduce toxicity after hepatic cancer irradiation.

AFP is associated with the liver tumor's prescence and it is the primary tumor marker for HCC[47,48] with 41-65% sensitivity[48]. In high-risk patients, It was stated that using AFP as a screening test can increase the number of early tumour detections and increase patient survival. [47]. AFP quantatitive level was lower in PLDR than CRT.This biochemical results agree with our pathology results.

The significant higher IL-6 serum level in CRT than PLDR group was accompined with vascular congestion , extravasation and balloning (is a particular type of hepatocyte damage that has been linked to more severe liver disease and an increased risk of complications from liver-related conditions). [49] In addition to the weight loss of the rats in CRT group. All these indicate that PLDR could alleviate the toxicity more than CRT.

It should be mentioned that the selection of 3- minutes interval breaks in PLDR was based on the work done by other earlier studies [29-33]. It was also stated by Ma et al, a 3 minutes interval can be more practical to keep the overall therapy session within 30 minutes[38].

Conclusion

Our in vivo experiments reveal that the PLDR treatment exhibits at least comparable tumor control when compared to conventional radiotherapy. PLDR technique can enhance the repair process of normal tissue damage and, therefore, lead to significantly less toxicity to normal tissue than conventional RT. We expect PLDR to be a viable modality for the management of liver cancers. In addition, one of the major advantages of the PLDR technique is that most radiation oncology facilities may adopt it without having to install new equipment or train more therapy staff.

Funding statement

This study didn't receive any funding support.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was carried out in compliance with a protocol approved by Scientific Research Ethics Committee of Ain Shams University (Code: ASU‐SCI/PHYS/2023/9/1).

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تأثير العلاج الإشعاعي بمعدل جرعة منخفضة متقطع على نموذج أورام الكبد في الفئران

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تهدف هذة الدراسة إلى التوصل لمدى فعالية علاج الإشعاع منخفض الجرعة المتقطع (PLDR) في العلاج الاشعاعي لسرطان الكبد ، وذلك بإجراء تجارب حية بواسطة حقن للفئران بهدف إصابة الكبد بأورام سرطانية باستخدام ثنائي إيثيل نيتروسامين (DENA) ، وللبدء في تنفيذ وتقييم تلك التجربة تم تقسيم الفئران إلى أربعة مجموعات وهم مجموعة سلبية غير مصابة ، ومجموعة ايجابية مصابة بأورام سرطانية بالكبد لم تتلقى علاج ، ومجموعة ايجابية تتلقي العلاج بطريق الإشعاع التقليدي (CRT) ، ومجموعةايجابية اخرى تتلقى العلاج الإشعاعي بطريقة (PLDR) ، وقد تلقت كلتا المجموعتين الأخيرتين المشعتين جرعة في حدود ٦ جراي للكبد باستخدام شعاع ٦ ميجا فولت من المسرع الخطي Varian–Unique. . وبالتزامن مع ذلك تم رصد وزن الجسم يوميًا وقياس حجم الورم للفئران باستخدام الأشعة المقطعية (CT) ، وأعقبنا ذلك بإجراء تحاليل نسيجية على أنسجة الكبد، وتقييم مستوى ألفا فيتوبروتين (AFP) وإنترلوكين ٦ .(IL–6) .وحسبما أسفرت النتائج التي توصلنا إليها تبين تقليل نمو الورم الكبدي بشكل كبير للفئران المتلقية للعلاج الإشعاعي مقارنة بالمجموعة غير المعرضة للإشعاع باحتمالية ($P < 0.05$) ، كما أظهرت تقنية PLDRأثاراً إيجابية ونتائج أفضل مقارنة بتقنية CRT ، حيث أظهرت مجموعة الفئران المتلقية للعلاج الإشعاعي بتقنية PLDR زيادة مستمرة في الوزن ، في حين أن مجموعة الفئران المتلقية للعلاج الإشعاعي بتقنية CRT أظهروا فقدانًا تدريجيًا في الوزن حتى اليوم الخامس ، كما أظهرت كلا المجموعتين انخفاضًا كبيرًا في مستوى AFP و IL–6 في السيرم مقارنة بالمجموعة الإيجابية . وكانت مدلولات تقديرات AFP و IL–6 في PLDR أقل من CRT وذلك باحتمالية احصائية (P=0.16و P=0.001) على التوالي ، كما كانت متوسطات مستويات AFP في السيرم ٠,٥٥٤ ±٠,١١، ٢,٥٢± ٠,٢٣، ١,٢٥٨± ٠,٣٩ و٠,٩٧٦± ٠,٣٥، ومستويات IL–6 كانت ١٦,٠١± ٢,٥، ٤٦,٩±٢,٩، ٢٧,٥±١,٥ و٢٢,٥±١,٠٣ للمجموعة السلبية والمجموعة الإيجابية ومجموعة PLDR ومجموعة CRT على التوالي ، وقد أظهر نتيجة الفحص النسيجي لمجموعة CRT ومجموعة PLDR إلى مزيد من تدهور خلايا الكبد مع مجموعة CRT ، بما يكون معه الناتج النهائي أنه يمكن التحكم في أورام الكبد باستخدام PLDR بنفس فعالية CRT ، مع ميزة إيجابية تتمثل في إظهار سمية أقل بكثير للأنسجة الطبيعية.