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Original Article

A Survey on the Possibility of Utilizing γH2AX as a Biodosimeter in Radiation Workers

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Abstract

Introduction
DNA damage is among the main consequences of radiation. Of many different classes of DNA damage, double-strand breaks are the most deleterious. Development of a sensitive biodosimetry method, which utilizes a detection material with a similar construction to the body, seems essential for monitoring radiation workers. In this study, histone H2AX protein was examined as a potential biodosimeter in radiation workers. Moreover, the presence of this protein after in vitro irradiation of blood samples was assessed simultaneously.

Materials and Methods
Blood samples from 46 radiation workers were analyzed in Golestan province, Iran. Meanwhile, two groups of blood samples (five blood samples in each group) were irradiated in vitro by doses of 1 to 0.2 Gy and 0.09 to 0.01 Gy from a ^{60}Co source, respectively. γH2AX level in lymphocytes was measured, using Western blot technique. ANOVA and Tukey's tests were performed, using SPSS version 16. The significance level was considered to be 0.05.

Results
The results of Western blotting for the identification of γH2AX protein in radiation workers were negative. However, γH2AX level in lymphocytes of two in vitro irradiated groups showed a significant correlation with the radiation dose ($P<0.0001$).

Conclusion
The results showed that γH2AX was a good indicator for acute or local exposure to ionizing radiation, while in chronically exposed individuals, including radiation workers, this protein was useless at least in autoradiography detection method. Regarding the presence of γH2AX protein in blood samples, which were irradiated in vitro at low doses, it can be concluded that this protein has powerful repair mechanisms.

Keywords: Biodosimetry; γH2AX; Radiation Worker

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1. Introduction

Today, application of radiation is an undeniable necessity in human life [1]. Many investigators have noted the significant effects of radiation on health. As the review of the history of radiobiology indicates, the biological effects of high-dose radiation were known after the discovery of X-ray and radioactivity.

However, the effects of low-dose radiation on human health are still unclear and under investigation [2]. In fact, the threshold dose, i.e., the dose above which radiation leads to adverse effects on the body, has not been yet established [3]. Nevertheless, the maximum allowed dosage for radiation workers is determined to be 20 mSv per year. According to the guidelines by the International Commission on Radiation Protection (ICRP), to avoid the possible adverse effects of radiation such as cancer and genetic diseases, the annual received dose to general populations should not exceed 1 mSv [4].

In vivo studies on radiation workers, who are occupationally exposed to low levels of radiation, could provide a more accurate threshold level [5]. Dicentric chromosome count in the metaphase spread of stimulated lymphocytes is a common gold standard for biological dosimetry [6]. This gold standard is specific to ionizing radiation and remains unchanged for several months after receiving ionizing radiation [6]. However, at least a 52-hour interval is required between the time of blood sampling and dose estimation, which is not ideal [6, 7].

DNA double-strand break (DSB) is one of the main consequences of exposure to ionizing radiation. DSB is arguably the most dangerous DNA lesion that could induce genomic and cancerous changes in cells and lead to mortality at high levels [8]. Recently, an altered protein has been suggested as a potential indicator for exposure to ionizing radiation, which is significantly associated with radiation-induced DSB [9].

Histone H2AX, a variant of histone H2A, becomes phosphorylated on serine residue 139 in response to radiation exposure, so-called gamma-H2AX, which signals the repair of DSBs [10]. Protein detection seems to be a suitable method for initial triage, as responses to radiation begin within a few minutes and remain stable for several days after exposure [11]. The presence of gamma-H2AX protein can be detected by sensitive, quantitative, or semi-quantitative methods [11].

Various human specimens including lymphocytes, mouth cells, and skin can be used for the detection of gamma-H2AX after ionizing radiation [12]. Despite the abovementioned points, no comprehensive study has shown the efficacy of gamma-H2AX as a reliable biological dosimeter in radiation workers. Therefore, in this complementary study, we examined gamma-H2AX as a biological dosimeter in radiation workers, employed by hospitals in Golestan province, Iran. Lymphocytes from individuals, chronically exposed to low doses of ionizing radiation from CT scan, radiation therapy, nuclear medicine imaging, and diagnostic radiology, were gathered and analyzed.

2. Materials and Methods

2.1. Study subjects

The test subjects included 46 radiation workers (27 females and 19 males), within the age range of 23-52 years, who were occupationally exposed to chronic doses of radiation while performing their routine tasks at diagnostic radiology units, nuclear medicine departments, and CT scan facilities in Golestan province, Iran. Their work experience ranged from 10.5 months to 27 years.

The control group comprised of 46 individuals (of the same sex and age as the test group), who were not occupationally exposed to radiation. None of the subjects in the control group were occupationally exposed to any carcinogenic agents other than ionizing radiation.

A questionnaire was developed to assess demographic information, work experience, previous radiation therapy, prior chemotherapy, smoking history, medication use, and prior history of receiving radiation exceeding the standard limit. In case such information was not accessible, the subject was excluded from the study.

The peripheral blood of subjects was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). The presence of γH2AX in peripheral blood lymphocytes was assessed at the Clinical Biochemistry Laboratory of Golestan University of Medical Sciences. Lymphocytes were separated from other blood components by ficoll solution at 2500 rpm within 10 min. The cells were transferred to another tube and washed at 2500 rpm for 5 min three times in cold phosphate-buffered saline (PBS) solution.

2.2. In vitro irradiation

At this stage, blood samples were collected from a healthy subject, based on the mentioned questionnaire. The blood samples were distributed among several aliquots in plastic tubes. Three blood samples were irradiated at each dose at room temperature using a ^{60}C0 source. The absorbed dose by blood samples was determined at 1, 0.8, 0.6, 0.4, 0.2, 0.09, 0.07, 0.05, 0.03, and 0.01 Gy with a rate of 67.55 cGy/min.

The calculated irradiation time ranged from 4.88 to 0.05 min. Lymphocytes were separated from other blood components by ficoll solution at 2500 rpm within 10 min. The cells were transferred to another tube and washed at 2500 rpm for 5 min three times in a cold PBS solution.

2.3. Extraction of cellular proteins

The lymphocytes in tubes were added to lysis buffer, containing 5% sodium dodecyl sulfate (SDS), 1 ml of Triton X-100, 100 mM of Tris-HCL (pH=7.4), 150 mM of NaCl, 5 mM of EDTA, 5% sodium deoxycholate, and 10% glycerol. The obtained solution was added to 10 μl/ml of phosphatase inhibitor cocktail (Sigma, p004) and protease inhibitor cocktail (Sigma, p8340). The protein samples were quantified by Pierce BCA Protein Assay Kit (Thermo, 23227), using bovine serum albumin (BSA) to form a standard curve.

2.4. Western blot analysis

Proteins are normally separated based on their molecular weight in SDS-PAGE electrophoresis. In our experiment, we used 15% gel, and then, 5x sample loading buffer, containing 10% SDS, 0.5% bromophenol blue, 60 mM of Tris-HCI (pH 6.8), 50% glycerol, and 14.4 Mm of 2-mercaptoethanol, was added to each tube and subsequently boiled for 5 min.

For blots, 100 μg of protein samples was loaded in each well. The sample proteins were separated on a 15% polyacrylamide gel and transferred to nitrocellulose membranes (Thermo) for 48 min at 110 V, using transfer buffer (15.6 Mm of Tris-base, 120 Mm of glycine, and 10% methanol, pH=8.4). The blots were incubated in 3% skim milk in PBS-T (0.05% Tween-20 at room temperature for 2-3 hrs on a rotating platform).

The blots were incubated overnight on a rotating platform at 4 °C in anti-phosphorylated histone H2A (Millipore, 05-636), with the dilution of 1:1000 in 3% skim milk/PBS-T. The blots were rinsed three times with BPS-T for 5, 19 and 15 min, respectively at room temperature on a rotating platform. They were then incubated with Goat Anti-Mouse IgG and horseradish peroxidase-conjugated secondary antibodies (Millipore, 12-349) at a dilution of 1:2000 in 3% milk/PBS-T for 1-2 hrs at room temperature on a rotating platform.

The blots were washed as before and exposed to autoradiography film after incubation for various durations (typically 10 to 15 min) with enhanced chemiluminescence reagents, as described by the manufacturer (Amersham ECL Biosciences, RPN2235). The films were finally developed and fixed for scanning and densitometry.

2.5. *Statistical analysis*

Statistical tests were performed using SPSS version 16.0. One-way analysis of variance (ANOVA) was used to determine any statistical differences in the level of γH2AX, induced by gamma irradiation in lymphocytes. Also, Turkey's test was performed for mutual comparison between the groups. In case the data were not normally distributed, Kolmogorov-Smirnov test was performed in each group. Spearman's correlation test was also performed to evaluate the relationship between radiation dose and γH2AX protein. P-value less than 0.05 were considered statistically significant.

3. Results

3.1. *Western blot analysis of radiation workers and the control group*

After determining the total protein concentration by BCA method, 100 μg of cellular protein from each sample was used for Western blot analysis (100 μg of protein was loaded in each gel well). The results of Western blotting for the identification of γH2AX protein in radiation workers and the control group showed no protein bands.

In order to evaluate the sensitivity and accuracy of the method, we directly irradiated the blood samples by a ^{60}Co source; the samples along with the specimens were added to additional wells. γH2AX band corresponding the direct irradiation of blood samples (along with other specimens showing no protein band) is depicted in Figure 1. Moreover, we used beta-actin loading control as shown in Figure 2.

Figure 1. The results of Western blot analysis of cellular γH2AX protein in radiation workers and the control group (on the left)
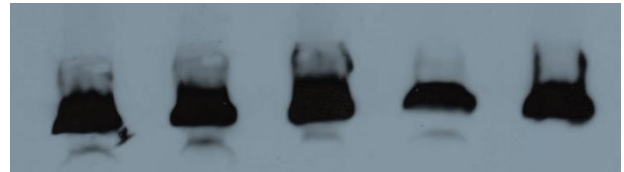

Figure 2. The results of Western blot analysis of cellular beta-actin protein

3.2. *In vitro irradiation*

To evaluate the relationship between radiation dose and formation of γH2AX protein, ten different radiation doses were selected in two equal groups (receiving 1-0.2 Gy and 0.09 - 0.01 Gy, respectively). At this stage, three samples were irradiated in vitro. The results of Western blot analysis of γH2AX in human lymphocytes after in vitro irradiation are shown in Figures 3 and 4.
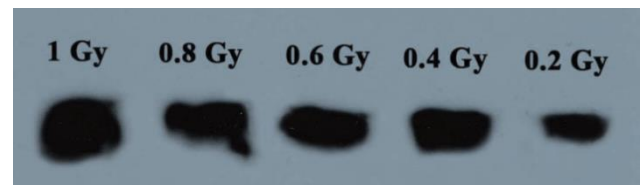

Figure 3. The results of Western blot analysis of γH2AX protein, induced by radiation doses ranging from 1 to 0.2 Gy
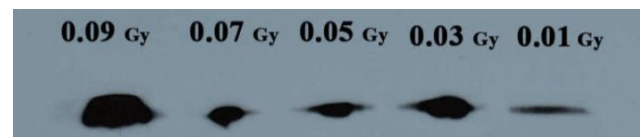

Figure 4. The results of Western blot analysis of γH2AX protein, induced by radiation doses ranging from 0.09 Gy to 0.01 Gy

3.3. *Densitometry of Western blot bands*

For the quantitative evaluation of in vitro irradiation, Western blot bands were scanned and analyzed using Image J software(National Institutes of Health (NIH) Version 1.49p 28 February 2014). Color intensity and the area under density profile were scored. We aimed to convert the profile areas and band intensities to numeric quantities for statistical analysis, making comparisons, and definitive conclusions.

It should be noted that the calculated digits by Image J software are only representatives of a

certain group of profiles without any other significant meaning. In other words, these numbers do not describe the level of proteins in bands in milligram or any other unit. ANOVA test of densitometry profiles corresponding to 1-0.2 Gy and 0.09-0.01 Gy groups showed a significant difference (P<0.0001) in the density of protein bands. The obtained results are shown in tables 1 and 2.

Also, the results of Tukey's test for the mutual comparison of groups showed that in the first group, all doses (0.8 Gy, 0.6 Gy, 0.4 Gy, and 0.2 Gy) were significantly different from 1 Gy (P<0.0001). Moreover, doses of 0.8, 0.6 and 0.4 were significantly different from 0.2 Gy.

However, radiation doses of 0.6, 0.8 and 0.4 Gy were not significantly different from one another.

In the second group, Tukey's test showed that all doses (0.09 Gy, 0.07 Gy, 0.05Gy, and 0.03 Gy) were significantly different from 0.01 Gy (P<0.0001), while radiation doses of 0.09 Gy, 0.07 Gy, 0.05 Gy and 0.03 Gy were not significantly different. However, Spearman's correlation test showed a significant association between radiation dose and γH2AX protein level in the first group (r=0.941, P<0.0001) and the second group to a lesser extent (r=0.595, P<0.0001). The data are shown in figures 1 and 2.

Table 1. ANOVA test results of densitometric profiles of 1-0.2 Gy group

Significance level	Mean	SD	Radiation dose (Gy)
	28.4130	1.38689	1
	21.3927	.64996	0.8
P<0.0001	19.7438	.25132	0.6
	19.0933	.85381	0.4
	11.6903	1.24250	0.2

Table 2. ANOVA test results of densitometric profiles of 0.09-0.01 Gy group

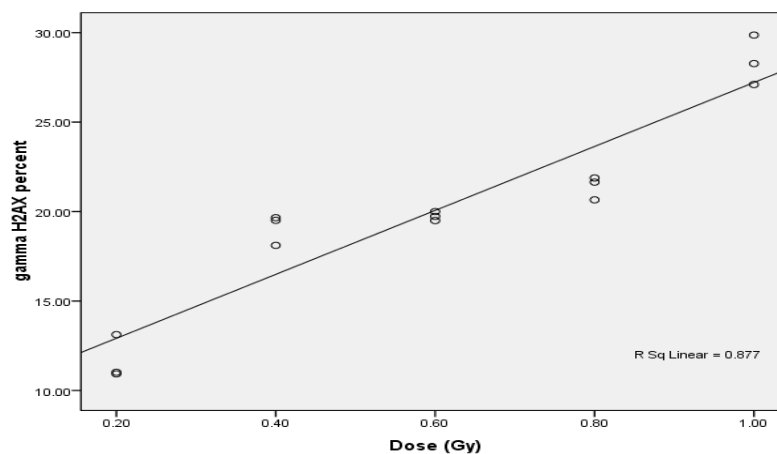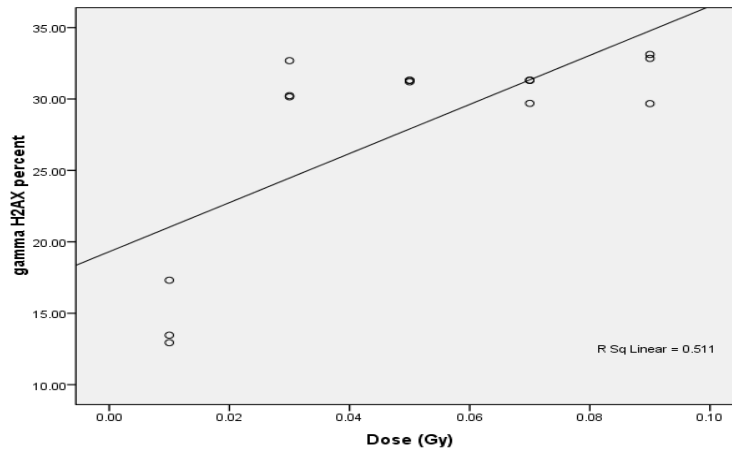| Significance level	Mean	SD	Radiation dose (Gy)
	31.8790	1.91370	0.09
	30.7733	.93398	0.07
P<0.0001	31.2723	.06165	0.05
	31.0223	1.43794	0.03
	0.01	2.38803	14.5710

Figure 1.The correlation between γH2AX and radiation dose in the 1-0.2 Gy group, calculated by the Spearman's correlation test related to the densitometry profiles

Figures 2. The correlation between γH2AX and radiation dose in the 0.09-0.01 Gy group, calculated by Spearman's correlation test related to densitometry profiles

4. Discussion

Considering the increasing medical use of radiation in radiology, nuclear medicine, and CT scan, particular attention should be paid to the radiation dose. Radiation workers in therapeutic and diagnostic facilities, despite the appropriate use of personal protective devices and regulations, receive low exposure levels within a long period [13].

Cancerous and genetic effects of radiation are major health concerns after chronic exposure in occupationally exposed individuals, while their underlying mechanisms remain largely unclear [14, 15]. Generally, the results of previous human studies have showed that the received doses are within the annual permitted range Previous studies suggest that monitoring of radiation workers should not be solely based on physical dosimetry [13]. In fact, it is necessary to use biological indicators which could provide individual radiation damages [17]. Development of a simple, rapid and efficient method for biological dosimetry seems essential in cases of acute or chronic exposure [18].

A linear relationship has been documented between the number of DSBs and γH2AX protein at different ionizing radiation doses [11]. This may be a potential source for the formation of chromosomal aberrations induced by radiation [19]. According to the results of this study, in radiation workers, who were occupationally exposed to chronic doses of radiation, no band of γH2AX protein was identified. This may be due to a robust and efficient repair system for DSBs at chronic radiation exposures [4]. In fact, at low doses of radiation received by workers, there is enough time for the repair of DSBs, in comparison with sudden received doses [3-20].

Contrarily, several studies have shown that chromosomal damages are more significant in radiation workers than the general population [21, 22]. On the other hand, DNA may be misreported instead of repaired in chronic radiation exposure, which may lead to chromosomal aberrations [12].

Main DNA damages induced by ionizing radiation include DSBs, single-strand breaks (SSBs) and DNA base damages [4-12] ; the ratio of SSB to DSB was estimated to be 20:1 [12], especially in low linear energy transfer radiation. However, SSBs could not lead to γH2AX foci formation [23]. In fact, the absence of γH2AX protein band in radiation workers does not indicate genomic stability and SSBs may be present. On the other hand, DSB does not always lead to γH2AX foci formation [24]. Rapid and complete disappearance of foci may also not be correlated with the repair of DSBs. Finally, the higher activity level of phosphatases may lead to the disappearance of γH2AX in DSBs [19-25].

There are two pathways for the repair of DSBs in mammalian cells, known as nonhomologous end joining (NHEJ) and homologous

recombination (HR). HR is the selected pathway for the repair of S and G2 phases of cell cycle; in this pathway, misrepair rarely occurs . Over 99% of cells in the human body (such as the sample in this study) are in G0 and G1 phases of cell cycle and the HR pathway in these cells is suppressed. Therefore, DSBs are repaired mostly in the NHEJ pathway, which can be prone to error [26].

Moreover, a significant correlation was found between irradiation dose and formation of γH2AX protein under in vitro conditions. Similar results have been obtained in other studies. Our results were in agreement with a study by Redon et al., who irradiated blood samples by doses of 5 to 0.2 Gy in ex vivo conditions; they found a linear response to this dose range. [1] Additionally, Havelek et al. aimed to determine whether phosphorylation of histone H2AX could be used as an indicator of received gamma radiation dose after whole-body irradiation of rats [17]; their results were also in agreement with our findings.

In this regard, Lefevre et al. examined the presence of γH2AX foci in isolated peripheral blood lymphocytes in ex vivo gamma radiation (0.02-2 Gy). They concluded that γH2AX can be a useful indicator, particularly for the classification and detection of minimal received radiation doses [7]. All these studies showed a significant relationship between radiation dose and γH2AX protein level.

5. Conclusion

The results showed that γH2AX measurement is effective for monitoring individuals with acute or local exposure (such as radiological accidents) and patients undergoing radiation therapy. However, γH2AX showed no sensitivity to low chronic exposure in occupationally exposed individuals. Strong DNA repair mechanism, detection method of protein bands, and autoradiography may be the main underlying causes.

Acknowledgements

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