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# Development of methemoglobin-based biological dosimetry in gamma–irradiated mice

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► ***Original article***

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## ABSTRACT

***Background***: A new biological dosimeter based on methemoglobin level was developed in this study. ***Materials and Methods***: Methemoglobin level in erythrocytes from mice subjected to γ rays from a $^{60}$Co source was detected using the methemoglobin kit. The dose range was from 0.5 to 8 Gy and the dose rate was 0.5 Gy/min. ***Results***: The results demonstrate that methemoglobin level increases with increasing dose. The detection limit based on methemoglobin has a lower limit of dose estimation of about 1 Gy. The high levels of methemoglobin are maintained for at least 28 days, and the maximal increase of methemoglobin observed occurs at about 30 min after γ irradiation. The relationship between dosage and the increased methemoglobin level can be expressed by a linear quadratic equation of $y = -8.75\ x^2 + 168.09\ x + 32.66$, with the correlation coefficient, r, equal to 0.96. The best suggested time for blood collection is up to 1 day after γ irradiation. The doses absorbed by mice as estimated from the use of the dose-response relationship were close to the blind doses of 1, 2, 4 and 8 Gy. ***Conclusion***: Methemoglobin is a quick, simple, and precise biomarker for the early assessment of the absorbed dose in mice.

***Keywords:*** *Biological dosimetry, methemoglobin, gamma irradiation, mouse.*

## INTRODUCTION

Worldwide, the application of nuclear technologies is rapidly increasing. In spite of strict regulations and safety measures, radiation/nuclear accidents or unplanned radiation exposures may occur. In these scenarios, it is unlikely that physical dosimeters will be available for dose measurement to aid clinical management of mass casualties. A biological dosimeter is a detectable variation of a biological parameter altered after radiation exposures, which can be used for the quantification of the absorbed dose of a large number of people after an unplanned exposure [(1)]. Biological dose assessment can help develop a treatment strategy for the radiation victims rapidly after a radiation catastrophe.

The biodosimetry scientific community has established two research directions in biological dosimetry based on the requirements for clinical management of mass casualties after a radiation catastrophe: (a) definitive, rapid and high-throughput radiation dose assessment and (b) triage-type radiation dose assessment [(2)]. The definitive, rapid and high-throughput radiation dose-assessment bioassay detects cytogenetic chromosomal aberrations of peripheral blood lymphocytes. These aberrations include chromosomal dicentrics [(3)], premature condensed chromosomes (PCCs) [(4, 5)], and the micronucleus [(6)]. The triage-type radiation dose



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assessment bioassay that detects radiation-responsive molecular biomarkers is still in its infancy as a scientific discipline. These biomarkers include proteins [7-9], gene expression [10-12], DNA mutations [13], and enzyme logic analysis based on biomolecular information processing [14]. The methods mentioned above, especially the cytogenetic assays, however, are laborious and time-consuming. Hence, a quick, simple, and precise method to assess biological radiation dose is still required.

Ionizing radiation generates many kinds of free radicals in organisms. Unpaired electrons of these free radicals may induce gain or loss of electrons to metal ions, which can result in the alteration of the valence of these metal ions. Due to the radiation sensitivity of the ionic valence of metal trace elements, the altered ionic valence may be used to assess the biological radiation dose. Our previous results demonstrate that serum iron level increases with increasing gamma dose, and serum copper level decreases with increasing gamma dose. Both trace elements can be used to rapidly and accurately assess the biological dose of mice at an early stage of radiation exposure [15, 16]. In living organisms, iron exists in two states of the ionic valence: the ferrous ion and ferric ion. The ferrous ion is mainly used to synthesize the non-protein moiety of haemoglobin, i.e. the haeme group, which consists of the ferrous ion and pyrrole molecules. It is well known that the function of haemoglobin is to transport oxygen from the lungs to the tissues. In addition to oxygen carriage, haemoglobin is also involved with other physiological functions, such as inflammation and vascular regulation [17, 18]. The latter two functions are performed by a cyclic system between the ferrous state of haemoglobin and the ferric state of methemoglobin. Methemoglobin is a form of haemoglobin, which contains the ferric ion [19]. In the normal physiological state, methemoglobin is formed by auto-oxygenating the heme iron of oxygenated haemoglobin. The rate of auto-oxygenation is about 3 % per day [20]. Free radicals generated by ionizing radiation in an organism may take one electron from the ferrous ion of oxygenated haemoglobin to form methemoglobin, a metalloprotein. In this study, the authors used methemoglobin to estimate the absorbed dose to an organism.

A methemoglobin-based biological dosimeter for mice has been established in this work. First, we detected the concentration of methemoglobin in mice irradiated with different gamma doses. Second, we investigated the dose response of methemoglobin according to its concentration change after γ irradiation. Third, we explored the maintenance of the high levels of methemoglobin after radiation exposure. Finally, a biological dose assessment in mice was performed using the established dose-response relationship.

## MATERIALS AND METHODS

### *Animals*

Eight-week-old male mice (ICR, specific pathogen free) were purchased from the Animal Center of Nantong University (Nantong, Jiangsu, China) and were housed in cages in an animal room for 1 week under the following conditions: temperature was controlled at 23 ± 2 °C, relative humidity was 55 ± 15 %, 12 air changes per hour, and a 12 h light/dark cycle. The mice had free access to tap water and to a pelleted commercial laboratory animal feed. The animal feed is prepared from flour, wheat bran, yeast extract, corn meal, bone meal, sorghum, and fishmeal. This study was performed in accordance with the Ethical Guidelines for Animal Experiments established by the Ministry of Science and Technology of the P. R. China.

### *Radiation exposure*

Whole-body γ irradiation was performed with a $^{60}$Co source (Gaotong Isotope Co., Chengdu, Sichuan, China). The gamma source is unilateral. The mice were placed in a ventilated Plexiglass cage, restrained with rubberized tapes, and irradiated in groups of six. The absorbed doses were 0, 0.5, 1, 2, 4 and 8 Gy, respectively. The dose rate was 0.5 Gy/min, which was selected to achieve good control over the accuracy of the low dose (0.5 Gy). The source-to-target distance





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was 0.75 m. Dosimetry was performed on a regular basis with a 0.6 $cm^3$ Farmer Ionization Chamber (Type 30010) which was connected to a dosimeter (Unidos, PTW-Freiburg Co., Freiburg, Germany). The chamber was placed next to the Plexiglass cage for irradiation.

### *Determination of methemoglobin concentration*

Peripheral blood was collected into a heparinized tube from the peri-orbital sinus of gamma-irradiated mice. The concentration of methemoglobin in the erythrocytes was measured using Methemoglobin kit (Jiancheng Inc., Nanjing, Jiangsu, China). Blood sample (10 µl) was mixed with diluent (2.5ml) of reagent 1 (for haemoglobin detection) for 5 min. The optical density of the mixed liquid was read at 540 nm using a microtiter plate reader (Bio-Rad Co., Hercules, CA, USA). The rest of blood sample (50 µl) was mixed with diluent (2.5ml) of reagent 2 (for methemoglobin detection) for 5 min. The optical density of the mixed liquid was read at 630 and 602 nm, respectively. The concentration of methemoglobin was calculated using the following formula:

$$C_{mHb} = \frac{A_{630\,nm} - 0.14 \times A_{602\,nm}}{A_{602\,nm} \times 1.67} \times 100\,\% \times A_{540\,nm} \times 367.7$$

### *Statistical methods*

Each dose group included six mice, except the group for dose assessment, which included four mice. Each blood sample was in triplicate detection. The data were presented as mean ± standard deviation and processed with OriginPro 8.0. One-way analysis of variance (ANOVA) and Dunett's test were applied to analyse the data. *P* values less than 0.05 were considered statistically significant. The *t* distribution was used to calculate the 95% confidence interval of the dose assessed.

## RESULTS

### *Dose response of methemoglobin*

Methemoglobin level in mice 30 min after whole-body γ irradiation was measured. Figure 1a presents the concentration of methemoglobin in mice irradiated with different γ doses ranging from 0.5 to 8 Gy. The concentration of methemoglobin in non-irradiated mice was 40.12 ± 5.21 µg/ml; methemoglobin increases with increasing dose, and the methemoglobin level reaches 790.63 ± 38.92 µg/ml when the dose is 8 Gy. The concentration of methemoglobin corresponding to 0.5 Gy was not significantly higher than that of non-irradiated controls; however, γ doses equal to or above 1 Gy induced significantly higher levels of methemoglobin compared to that of non-irradiated controls. Therefore, the detection limit based on methemoglobin in gamma-irradiated mice was about 1 Gy. By analysing the concentration of methemoglobin and the corresponding γ doses, a linear quadratic equation of $y = -8.75\,x^2 + 168.09\,x + 32.66$ with a correlation coefficient of 0.96 ($p \leq 0.01$) is obtained. The linear quadratic fit to the data points requires the exclusion of the point for 0.5 Gy because it is not significantly higher than that of the non-irradiated controls. Figure 1b presents the linear quadratic curve based on the concentration of methemoglobin versus γ dose.

### *Maintenance of high levels of methemoglobin in gamma-irradiated mice*

Methemoglobin levels in mice at 30 min, 2 h, and 12 h after 8 Gy of γ radiation, and on days 1, 7, 14, 21 and 28 thereafter were recorded to study their maintenance. This radiation dose of 8 Gy is about the LD50/30 of the ICR mice [21, 22]. Figure 2 presents the changes in the methemoglobin level from mice as a function of time after 8 Gy of γ irradiation. The methemoglobin level increased to a maximum 30 min after γ irradiation and this level stayed constant for about 1 day. After day 7, the concentration of methemoglobin decreased slightly, but was then maintained at a steady level during days 14-28. Figure 2 shows that the high levels of methemoglobin in mice after whole-body γ radiation of 8 Gy were maintained for at least 28 days.

### *Biological dose assessment using the dose response of methemoglobin*

The concentration of methemoglobin in mice 30 min after γ irradiation is higher than that





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observed 1–28 days post-irradiation. Therefore, the dose response of methemoglobin 30 min after γ irradiation was adopted to assess the absorbed dose of mice. The blinded method was used in the experiment. The blinded doses were 1, 2, 4 and 8 Gy, respectively. Four mice were included in each blinded dose group. Blood was collected from the mice 30 min after γ irradiation. Table 1 presents the predicted absorbed doses of mice based on the linear quadratic relationship obtained for methemoglobin 30 min after γ irradiation. The absorbed doses assessed were generally close to the "blind test" doses of 1, 2, 4 and 8 Gy. The ranges of the 95% confidence intervals of the doses assessed are 1.08, 1.38, 1.84 and 1.84, respectively.

**Table 1.** Methemoglobin-based dose assessment (30 min after γ irradiation).

| Exposed mice | The concentration of methemoglobin (μg/ml) | The absorbed dose assessed (Gy) | 95 % confidence interval | Blind dose (Gy) |
|---|---|---|---|---|
| 1 | 178.38 ± 3.63 | 0.91 | 0.30 ~1.38 | 1 |
| 2 | 155.35 ± 6.25 | 0.76 | | |
| 3 | 103.32 ± 3.49 | 0.43 | | |
| 4 | 232.02 ± 6.12 | 1.27 | | |
| 5 | 328.50 ± 4.58. | 1.96 | 1.48 ~ 2.76 | 2 |
| 6 | 425.13 ± 6.83 | 2.72 | | |
| 7 | 308.24 ± 5.86 | 1.81 | | |
| 8 | 335.17 ± 3.99 | 2.01 | | |
| 9 | 563.05 ± 9.69 | 3.98 | 3.16 ~ 5.00 | 4 |
| 10 | 497.56 ± 12.73 | 3.35 | | |
| 11 | 515.92 ± 11.37 | 3.52 | | |
| 12 | 650.31 ± 12.34 | 4.65 | | |
| 13 | 815.95 ± 7.56 | 7.95 | 6.97~8.81 | 8 |
| 14 | 782.80 ± 9.21 | 7.05 | | |
| 15 | 823.14 ± 11.48 | 8.22 | | |
| 16 | 826.39 ± 12.84 | 8.36 | | |

The concentration of methemoglobin is presented as mean ± standard deviation. The *t* distribution was used to calculate the 95% confidence interval.

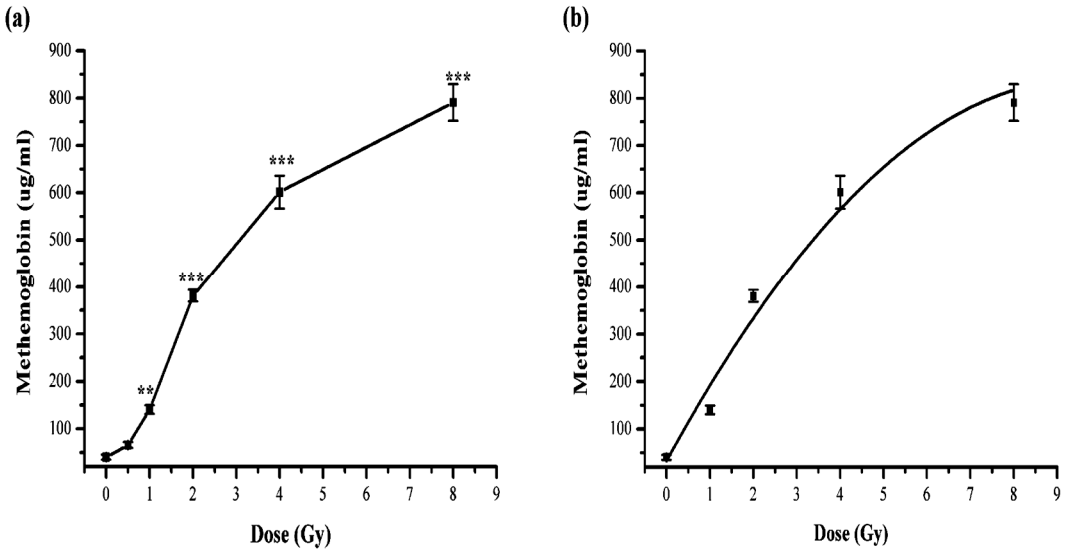


**Figure 1.** The dose response of methemoglobin in mice 30 min after γ irradiation. (a)The concentration of methemoglobin in mice irradiated with γ rays ranging from 0 to 8 Gy. Error bars indicate standard deviations (n = 6). (*) as compared to 0 Gy (** $P < 0.01$; *** $P < 0.001$). (b) The linear quadratic relationship ($y = -8.75\,x^2 + 168.09\,x + 32.66$) between the concentration of methemoglobin and the γ dose.





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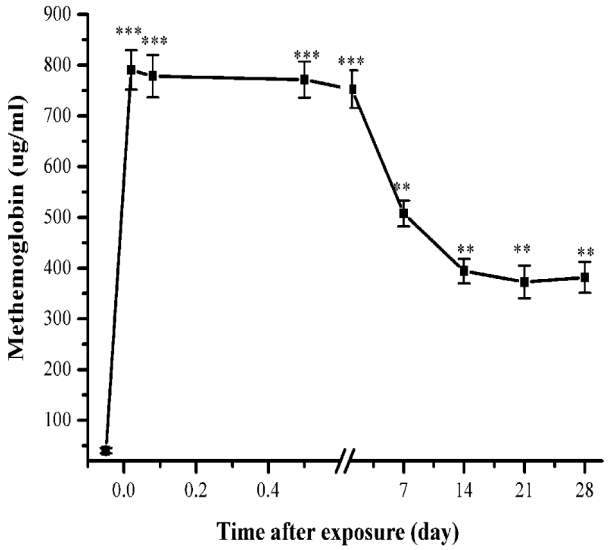


**Figure 2.** Maintenance of increase in the methemoglobin level from mice irradiated with 8 Gy of γ dose. Error bars indicate standard deviations (n = 6). (*) Compared to that before γ irradiation (**$P < 0.01$; ***$P < 0.001$).

## DISCUSSION

Methemoglobin, an oxidized metalloprotein, was explored as a biological dosimeter in this work. Methemoglobin level increases in the gamma-irradiated mice and this increase is dose -dependent (figure 1a). These results clearly demonstrate that methemoglobin is radiation sensitive. The increase of methemoglobin in the gamma-irradiated mice is due to free radicals generated by γ irradiation: these free radicals or their secondary products attack the heme iron of haemoglobin and capture an electron from the haeme iron to form methemoglobin. The mechanism of this kind of acquired methemoglobinemia [23, 24] is different from that of congenital methemoglobinemia. The latter is a hereditary disease caused by a deficiency of NADPH-dependent methemoglobin reductase [25, 26]. The concentration of methemoglobin in non-irradiated mice is about 40.12 μg/ml (figure 1a). This background value derives from the auto-oxidization effect of reactive oxygen species generated during the metabolic process of organisms.

Maintenance of the level of a biomarker is highly desirable and an important parameter for a biological dosimetry. The increased levels of methemoglobin in mice induced by 8 Gy of γ irradiation were maintained for about 28 days (figure 2). The methemoglobin level is maintained for a shorter duration than the cytogenetic biomarkers, such as the dicentric and the micronuclei [27, 28]. This difference might be due to the relatively short-term effects of free radicals. The change of methemoglobin after γ irradiation has a similar profile to that of serum iron [15]. This similarity may be attributable to free radicals attacking the same type of ion, i.e. the ferrous ion. We have not studied the effect of multiple doses on the production of methemoglobin in this study. However, we think that multiple doses might produce the same methemoglobin profile as that generated by 8 Gy of γ rays because serum iron, which also contains the ferric ion, has a similar profile of increase in mice irradiated with different γ doses [15]. According to the graph of the increase in methemoglobin level as a function of time, it is suggested that the best time for blood collection is up to 1 day after γ irradiation.

The dose-response relationship between dosage and methemoglobin level in mice 30 min after γ irradiation was established because methemoglobin increased maximally 30 min after γ irradiation. This linear quadratic relationship (figure 1b) is different from the linear relationship for serum iron 10 min after γ irradiation [15]. The lower limit of dose estimation based on methemoglobin level is about 1 Gy. As a radiation-responsive molecular biomarker, the lower limit of methemoglobin is higher than that of the dicentric chromosome (0.05 Gy), one of the definitive and rapid radiation dosimeters [2]. The lower limit of methemoglobin is also higher than that of serum iron (0.5 Gy) [15]. This difference might be attributable to the different location of these two substances; serum iron in the plasma may be more likely to come into contact with free radicals. The doses reconstructed according to the dose response curve (figure 1b) of methemoglobin are generally close to the "blind" doses of 1, 2, 4 and 8 Gy and the 95% confidence intervals of the reconstructed doses are all relatively small (table 1). These results indicate that methemoglobin can be used to precisely





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predict the absorbed dose in mice. The 95% confidence interval of the predicted doses based on methemoglobin is near those based on serum iron and serum copper [15, 16]. The 95% confidence interval is a parameter for data precision. The similarity between the 95% confidence intervals of the predicted doses indicates that these three biomarkers have similar precision in estimation of the absorbed dose in mice.

Methemoglobin levels are not affected by gender [29]. The detection of methemoglobin, which needs only about 60 µl of blood, is simple, and can provide the dose information in about 1 h. Therefore, although the maintenance time is shorter than that of dicentric chromosomes and the lower limit of dose assessment is higher, methemoglobin meets the basic requirements of the triage-type biological dosimeter, i.e. precision, stability, small sample volume, simplicity, and speed.

## CONCLUSIONS

Methemoglobin was first used to estimate the absorbed dose of irradiated mice. Methemoglobin was demonstrated to be a quick, simple, and precise biomarker for the early assessment of the absorbed dose. We suggest that these advantages make methemoglobin suitable for rapid mass triage after radiation events. However, like all assays, methemoglobin -based dose assessment has its own drawbacks and limitations. The concentration of methemoglobin is often influenced by some diseases and drugs. The diseases include congenial methemoglobinemia, congenital haemolytic jaundice, paroxysmal haemoglobinuria and enterotoxemia; the drugs are methylene blue, magnesium sulphate, nitrites, sulphonamides, acetanilide, etc. These factors must be carefully controlled if methemoglobin is to be used to assess the radiation dose.

Although methemoglobin-based dose assessment was performed in mice and in a controlled environment, and may not translate directly to humans, our studies have demonstrated that methemoglobin is a promising biomarker for triage-type dose assessment.

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***Conflicts of interest:*** *none to declare.*

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