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Research Article

Effect of Gallium-68 isotope injection on hemoglobin derivatives concentrations after instant injection and its recovery in male rabbits (*Oryctolagus cuniculus*)

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Abstract
Oxidizing effects of ionizing radiation are well established and almost understood. However, exposure to low doses of widely used isotopes may result in minor and hidden oxidative stress in some forms of hemoglobin. This formation alteration regarding the legends of hemoglobin's stereochemical function may play a role in hemoglobin dysfunction. There are limited studies related to the effect of gallium isotope injections. The study intends to find the effect of gallium-68 isotope injection on male rabbits. It was conducted on thirty-two male rabbits (*Orycytolagus cuniculus*) divided into Group I: control and Group II: animals exposed to gallium-68 isotope at a similar dose commonly used in diagnostic protocols for humans. Blood samples were collected twice: the first was after two hours of injection with isotopes and the second was after twelve hours of injection. A linear, four-mathematical-equations matrix based on the Lamber-Beer law was used to measure the concentration of different hemoglobin derivatives. Results revealed a significant elevation ($P<0.05$) of methemoglobin, the oxidized form of hemoglobin, two hours after injection (Total hemoglobin = 4.463 ± 0.83), but this effect was completely reversed after twelve hours. This concluded that even low doses of isotopes result in oxidation of hemoglobin that recovers shortly. Furthermore, the outcome of the study supports the healthcare centres to understand the effect of gallium-68 injections on animals.

Keywords: Derivatives, Gallium-68, Hemoglobin, Methemoglobin, Oxidative stress, Oxidation

INTRODUCTION

The term "gallium" refers to the natural mixture of gallium-69 and gallium-71(Ilan *et al*., 2020). Despite this fact, it is usually written as ^{71}Ga. Both isotopes, 69 and 71, are stable and have no radioactive properties (Velikyan, 2015). Gallium has two other widely-used, commercially radioactive isotopes, gallium-67 and gallium-68. 67Ga is a gamma emitter with a half-life of 3.3 days, while 68Ga is a positron emitter with a half-life of 68 minutes (Poeppel *et al*., 2011 and Eichendorff *et al*., 2014). Both isotopes have several medical applications. 67Ga, due to its long half-life, is used in a standard nuclear medical imaging method known as a gallium scan (Minamimoto *et al*., 2016). Because of ^{68}Ga's short half-life, it is utilized as a tracer in PET scans to reveal different tissue absorptions, resulting in diagnostic images (Minamimoto *et al*., 2016).The parent nucleotide, 68Ga, has a long half-life (270.95 days) that makes it easy to transfer to hospitals and acts as a source of the 68Ga isotope for nearly a year (Deppen *et al*., 2016 and Mu *et al*., 2013). Wild *et al*. (2013) note ^{68}Ga's ability to be safely stored at the application site and be prepared according to the usage rate by a low energy cyclotron (Ge-68/Ga-68).

The oxidative stress that many biological tissues suffer from is a situation in which the whole-body antioxidants and free radical concentration become unbalanced (Sarangarajan *et al*., 2017). Many studies showed that a body exposed to repeated oxidative stress processes always end in major chronic diseases, e.g., cancer, and diabetes (Lourenço *et al*., 2019). Under normal conditions, human cells produce antioxidants to reduce the oxidation stress of free radicals. But, due to the effects of some internal and external factors, this balance may become unstable(Liu *et al*., 2018). Of the factors affecting this situation, diet, environmental pollution, exposure to ionizing radiation, and certain lifestyles (Kumar *et al*., 2015) are the most well-known.

One of the most common ways ionizing radiation emitted from radioisotopes is absorbed into biological tissues is through the oxidation process that starts immediately after radiation application(Spitz and Hauer-Johnson, 2014). Oxidation of biological materials results in varying amounts of free radicals (Havas, 2017), depending on the type and intensity of the radiation, as well as the type of biological material itself. Studies have shown that the effects of free radicals on biological materials may persist even after direct radiation exposure is stopped(Yahyapour *et al*., 2018).

Hemoglobin derivatives are altered forms of hemoglobin that are different in the molecules' non-protein portion(Yahyapour *et al*., 2018). The oxidized forms of hemoglobin cannot perform hemoglobin's normal functions, such as carrying oxygen and carbon dioxide (Song *et al*., 2018). These forms of hemoglobin are considered non-functional forms that may be counted using traditional lab methods(Diaconu, 2009). Carboxyhemoglobin and sulfhemoglobin are hemoglobin derivatives combined with carbon monoxide, while methemoglobin is the form in which the iron atom is oxidized and changed from a ferrous state to a ferric state(Redmer *et al*., 2020). Methemoglobin is characterized by a very low oxygen affinity that makes it unable to bind with oxygen even at elevated concentrations. Spectrophotometrically, methemoglobin is characteristic by a new band present at 630 nm(Zwart *et al*., 1986).

Gallium isotopes are widely used in one of the most recent tumor diagnosis and follow-up modalities, the positron emission tomography (PET) scan (Minamimoto *et al*., 2016). This technique is based on the direct theory that hyperactive nuclei absorb much more illuminated substances. This elevated absorption can be used in imaging as these cells will appear as illuminated spots. 68Ga binds to somatostatin receptors (sstr2) with a high affinity(Attia *et al*., 2015; Amor-Coarasa *et al*., 2016; Lenzo *et al*., 2018). Gallium also binds malignant cells more readily than normal cells due to the excessive sstr2 receptors found on malignant cells(Poeppel *et al*., 2011).

As ^{68}Ga is a beta emitter (Valko *et al*., 2007; Morishita *et al*., 2018), it is used as a diagnostic tool relative to body weight, activity, and biological markers. The isotope is safely eliminated through healthy patient kidneys (Breeman *et al*., 2005). The most adverse effects of multiple applications of gallium are nausea, pruritus, and flashing with no definite contraindications. Regarding long-term and cumulative applications, research shows a higher cancer risk (Gulcin *et al*., 2020). Free radicals produced from direct exposure to the ionizing radiation emitted from 68Ga may play a role in elevating biological oxidative stress (Zwart *et al*., 1984).

The present work aimed to evaluate the oxidative stress and hemoglobin oxidation present at the low-dose 68Ga used in the commercial diagnosis of human diseases and to what extent the body's natural antioxidant mechanisms may cover these effects using the male rabbits, Oryctolagus cuniculus

MATERIALS AND METHODS

There were 32 male rabbits (*Oryctolagus cuniculus*) used in this study, all of whom were 3–4 months old and weighed 1.5 kg (±0.1 kg). Free access to drinking water was provided to the rabbits that received a conventional rabbit pelleted diet and libitum. The rabbits were separated into two groups: Group 1, the control group, which did not receive isotopic injections, and Group 2, which received Gallium-68 isotope irradiation (22 animals). Taif University. Kingdom of Saudi Arabia's ethics committee accepted the experiment's design. Gallium Ga-68 dotatoc (Rx) was utilized in a 30-

mL multidose vial containing 18.5–148 MBq/mL (0.5–4.0 mCi/mL) of the 68Ga isotope.

It was determined that the dosage should be 2 MBq/kg, which is in line with the commercially available isotopes and administered intravenously. In order to achieve optimal isotope absorption, the rabbits were hydrated with saline injections prior to the injections being given. The left ear vein was utilized to collect blood samples, which were then kept in heparinized tubes at 70 C until the analysis was performed using an appropriate syringe.

The dose was calculated as 2 MBq/kg, identical to the isotopes used commercially in human diagnostic protocols. An intravenous application was used. The animals were hydrated using saline injections to ensure adequate isotope absorption for 7 days before the injection. The evaluation of hemoglobin derivatives concentrations was carried out after two hours of injection to ensure that gallium had reached a majority of cells to monitor the oxidative stress effects properly. To study the recovery rate of the oxidative stress, another hemoglobin-derivatives-concentrations assessment was carried out after twelve hours on all animals in the second group and compared with the same control.

The derivative concentrations of hemoglobin were measured by a modified Beer-Lamber law(Van Kampen and Zijlstra, 1983). Spectrophotometrically, the four types of hemoglobin (oxyhemoglobin, carboxyhemoglobin, sulfhemoglobin, and methemoglobin) have characteristic pigments peaks. Four linear equations were obtained at 500, 569, 577, and 620 nm wavelengths. A matrix instant solution of the four equations was carried out to calculate each derivative concentration separately.

The millimolar extinction coefficients are presented in the following equations with the four unknown concentrations of hemoglobin pigments (CHb-O2, CHb-CO, CMet-Hb, and CHb-S).

$$A^{500} = 5.05\,C_{HbO_2} + 5.35\,C_{HbCO} + 9.04\,C_{Met.Hb} + 7.2\,C_{SHb} \quad \text{Eq. 1}$$

$$A^{569} = 11.27\,C_{HbO_2} + 14.27\,C_{HbCO} + 4.1\,C_{Met.Hb} + 8.1\,C_{SHb} \quad \text{Eq. 2}$$

$$A^{577} = 15.37\,C_{HbO_2} + 10.0\,C_{HbCO} + 4.1\,C_{Met.Hb} + 8.1\,C_{SHb} \quad \text{Eq. 3}$$

$$A^{620} = 0.24\,C_{HbO_2} + 0.33\,C_{HbCO} + 3.35\,C_{Met.Hb} + 20.8\,C_{SHb} \quad \text{Eq. 4}$$

The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 5.05 & 5.35 & 9.04 & 7.2 \\ 11.27 & 14.27 & 4.10 & 8.1 \\ 15.37 & 10.0 & 4.10 & 8.1 \\ 0.24 & 0.33 & 3.35 & 20.8 \end{bmatrix} \cdot \begin{bmatrix} C_{HbO_2} \\ C_{HbCO} \\ C_{Met.Hb} \\ C_{SHb} \end{bmatrix} = \begin{bmatrix} A^{500} \\ A^{569} \\ A^{577} \\ A_{620} \end{bmatrix} \quad \text{Eq. 5}$$

Mathematically, this is called a linear equation system that can be solved by a manipulation process. Gaussian elimination was used to calculate the different derivatives concentrations. Matrix calculation resulted in the following equations by which each concentration can be calculated separately.

$$C_{SHb} = \frac{A^{620} - 0.442293A^{500} + 0.1065519A^{569} + 0.0515769\,A^{577}}{18.895404} \quad \text{Eq. 6}$$

$$C_{Met.Hb} = \frac{9.0602343A^{500} - A^{577} - 2.6960235A^{569} - 35.295898\,C_{SHb}}{66.750821} \quad \text{Eq. 7}$$

$$C_{HbCO} = \frac{A^{569} - 2.2316831A^{500} + 16.074415\,C_{Met.Hb} + 7.9681188\,C_{SHb}}{2.330495} \quad \text{Eq. 8}$$

$$C_{HbO_2} = \frac{A^{500} - 5.35\,C_{HbCO} - 9.04\,C_{Met.Hb} - 7.2\,C_{SHb}}{5.05} \quad \text{Eq. 9}$$

Where A500, A569, A577, and A620 are the spectrophotometric absorbances of the hemoglobin solution at the wavelengths 500, 569, 577, and 620 nm, respectively.

Statistical analysis was carried out by SPSS 10.00 for Windows (SPSS Inc., Chicago, IL, USA). Student's T-test was chosen according to the experimental design in addition to a one-way analysis of variance test (ANOVA) for significance. Statistical significance was considered a p-value of less than 0.05.

RESULTS AND DISCUSSION

The relevance of understanding the harmful consequences of ^{68}Ga isotope coincides with its recently increasing medical utilization due to its ready availability, simple production, selective absorption by tumor cells, and amenability to one-step chelation(Kumar, 2020; Blower *et al*., 2021). These qualities have made it more pharmaceutically relevant than certain other isotopes, even in small medical institutions. This isotope has become scarcer due to an increase in PET scanning during the past decade (Blower *et al.,* 2021; Svedjehed *et al*., 2022). One advantage of ^{68}Ga isotope manufacturing and storage is the parent nuclide's long half-life (270.93 days). However, its widespread use should be accompanied by warnings about the dangers of prolonged exposure to ionizing radiation.

Toxic effects of ionizing radiation, like oxidative stress, have been widely documented. This stress is brought on by the radiation's direct contact with target macromolecules or by the radiolysis of water products, which can impact target molecules and spread to adjacent cells and tissues(Kumar *et al.,* 2021; Lin *et al.,*2018). Oxidative stress may be induced physiologically by various factors, including but not limited to ionizing radiation exposure. Radiation-induced cardiac disease was linked to oxidative stress caused by the ^{68}Ga isotope used in nuclear medicine, according to a recent study (RIHD). Chronic exposure to reactive oxygen species (ROS) can lead to oxidative stress, mostly de-

pendent on the body's capacity to detoxify ROS (Lin *et al.,*2018; Svedjehed *et al*., 2022). One of the most vulnerable molecules to oxidative stress is hemoglobin, because of its close association and interaction with oxygen molecules throughout the gas transmission. One hundred and forty one amino acids make up the alpha and 146 amino acids make up the beta hemoglobin transporters that work in tandem to transfer oxygen and carbon dioxide to and from cells in the blood's quaternary structure. The capacity of hemoglobin molecules to collect oxygen molecules that are more abundant in arterial circulation is known as hemoglobin's oxygen affinity(Kumar *et al.,* 2021; Lin *et al.,*2018). Atoms inside porphyrin ring influence hemoglobin's ability to bind to oxygen(Blower *et al.,* 2021). When hemoglobin is exposed to ionization, it undergoes changes in function due to this extremely sensitive state. When blood is exposed to radioactive isotopes, oxidative stress is one of the most prevalent reactions. The important redox system may require exogenous supplementation to rectify ROS's direct and indirect imbalance under high oxidative stress settings(Kumar,2020; Blower *et al*.,2021).

Data in Table 1 shows different hemoglobin derivatives concentrations in animals (Group II) injected with a gallium isotope after two hours to study the resultant oxidative stress following instant exposure to ionizing radiation. There was no significant effect of the sulfhemoglobin derivative. A significant increase in carboxyhemoglobin (2.1848%) was shown. A highly significant increase of methemoglobin (1.6939%) was revealed the derivative form in which the heme atom inside hemoglobin was oxidized and converted from a ferrous to a ferric state (Zwart *et al*., 1984). Hemoglobin oxygen affinity is dramatically affected when the heme atom's stereochemical characterizations are altered, in which case the hemoglobin loses its ability to perform its primary function, binding oxygen molecules. Spectrophotometrically, this derivative can be measured at a characteristic peak of wavelength 630 nm(Van Kampen and Zijlstra, 1983). However, low-dose exposure may result in low free radical production due to the direct oxidation caused by ionizing radiation. This means of evaluation may not be the most accurate method. Evaluation of the oxidized form of hemoglobin plays a more accurate assessment tool to define minor oxidative stress effects.

Table 2 shows different hemoglobin derivatives concentrations in animals injected with a gallium isotope after twelve hours to study the recovery of oxidative stress effects following exposure to ionizing radiation. An elevation of sulfhemoglobin recovered entirely after 12

Table 1. Hemoglobin different derivatives concentrations of animals received $_{68}$Ga as compared to control after two hours of injection

Haemoglobin derivates	Control		After two hrs. of injection	
	gm/dl	%	m/dl	%
S-Hb	0.031 ± 0.001	0.2192	0.037 ± 0.001	0.2558
Met-Hb	0.034 ± 0.002	0.2404	0.245 ± 0.023**	1.6939
Hb-CO	0.289 ± 0.031	2.0438	0.316 ± 0.016*	2.1848
Hb-O_2	13.786 ± 0.84	97.4964	13.865 ± 0.72	95.8653
Total Hb	14.14 ± 0.92	100	14.463 ± 0.83	100

* Significant P < 0.05, ** Highly Significant P < 0.01

Table 2: Hemoglobin different derivatives concentrations of animals received ^{68}Ga as compared to control after twelve hours of injection

Haemoglobin derivates	Control		After twelve hrs. of injection	
	gm/dl	%	m/dl	%
S-Hb	0.032 ± 0.001	0.2275	0.034 ± 0.001	0.2432
Met-Hb	0.037 ± 0.002	0.2631	0.033 ± 0.018**	0.2360
Hb-CO	0.316 ± 0.023	2.2470	0.337 ± 0.021	2.4105
Hb-O_2	13.678 ± 1.081	97.2623	13.576 ± 1.101	97.1101
Total Hb	14.063 ± 0.921	100	13.98 ± 0.992*	100

* Significant P < 0.05, ** Highly Significant P < 0.01

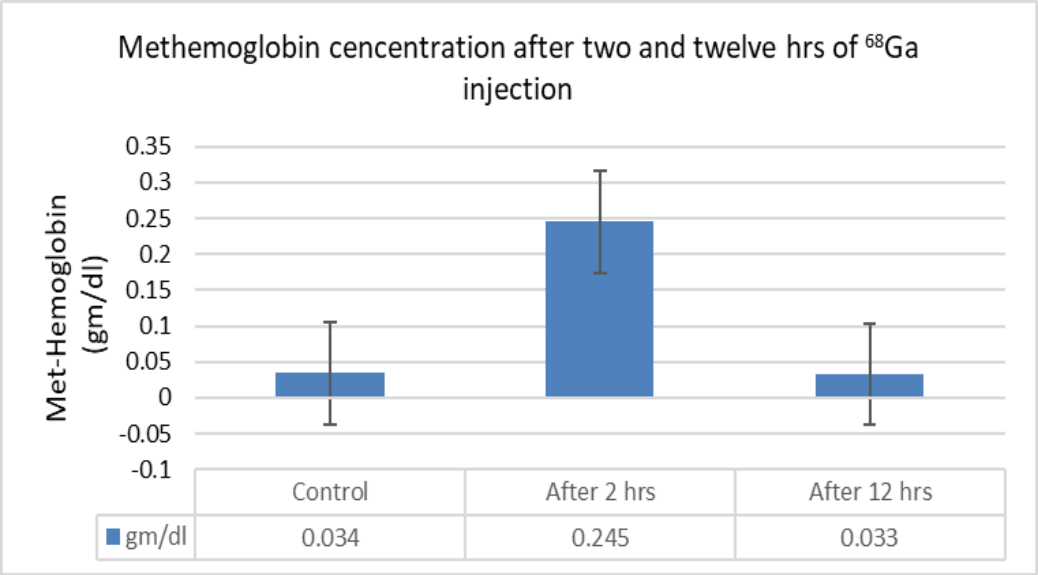

Fig. 1. *Methemoglobin concentration of animals after two and twelve hours of injection with 68Ga as compared to control*

hours. A fascinating finding was the highly-significant increase of the oxidized form of hemoglobin, methemoglobin, was completely recovered and returned to its normal levels. This rapid recovery rate verifies the body's antioxidative ability, even without antioxidant supplements (Hu *et al*., 2018). It means that the oxidative stress from this dose of ionizing radiation can be improved by the body's normal redox system. The existing studies state that antioxidants such as superoxide dismutase and glutathione persoxidase play an essential role in scavenging any free radicals formed due to oxidation (Ighodaro and Akinloye, 2018). On the other hand, the outcome of the present study confirms the outcome of the studies (Tieu *et al*., 2019; Gulcin, 2020; Redmer *et al*., 2020; Song *et al*., 2018) that describes the determination of hemoglobin derivatives. In addition, this study reveal that antioxidants are involved in reduction reactions, so their concentrations are reduced when oxidative stress is found. Therefore, the level of compensation between how many antioxidants are present and how many free radicals are formed becomes the main factor that controls in which direction the reaction will take place

In the present study, the value of methemoglobin concentration may be considered a reflection of free radical formation. This formation rate was significantly elevated Met-Hb of 2.41% in a short time after injection (Fig. 1). However, this adverse effect was significantly repaired 12 hours after injection, with no additional necessary supplements. The ^{68}Ga with a low dose of exposure always used in diagnosis had a very low oxidation effect on hemoglobin regarding the alteration in the magnetic status of the heme atom to be in ferric state instead of ferrous state. This methemoglobin derivative of hemoglobin can be recovered by the natural antioxidant's activity over time, measured after 12 hrs.

Conclusion

The outcome of the present study shows that the lower doses (18.5–148 MBq/mL) of ^{68}Ga isotope exposure caused minor oxidative stress due to its ionizing radiation emission on male rabbits, *O. cuniculus*. The rabbits injected with Gallium-68 had more excellent rates of autoxidation than rabbits that had not received protective antioxidant dosages. The outcome suggests the exposure to minor oxidative stress. It is also possible to examine the activity of naturally occurring bodily antioxidants to see whether either of these antioxidants can serve as therapy to protect cells from oxidative stress. The study findings' outline the constant occurrence of oxidation and reduction of hemoglobin molecules. It indicates the importance of preserving proper functional derivatives that enables hemoglobin to continue its regular tasks. Furthermore, the findings can be improved with the support of the natural oxidant activity of the body.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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