Interdiscip Toxicol. 2020; **Vol. 14**(2): 33–37.
doi: 10.2478/intox-2021-0006

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Short-Term Effects of Iodinated Radiographic Contrast Media on Red Blood Cells: Morphology, Osmotic fragility, and Hemolysis

Benjamaporn SUPAWAT [1,2], Singkome TIMA [3], Suchart KOTHAN [1,2], Montree TUNGJAI [1,2]

[1] Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand,
[2] Center of Radiation Research and Medical Imaging , Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand
[3] Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

ITX140221A01 • Received: 15 November 2019 • Accepted: 21 April 2021

ABSTRACT
Iodinated radiographic contrast media (IRCM) is currently being used in radiography for disease diagnosis. The aim of the present study was to examine three IRCMs (i.e., iohexol, iopamidol, and iodixanol) in terms of their impact on morphology, osmotic fragility, and hemolysis of red blood cells. Blood was shortly treated with IRCMs (1, 5, 10, and 50 mgI/ml) at 37 °C for 5 minutes. The morphology of red blood cells was then observed under a light microscope and the number of burr cells (echinocytes) was recorded. The osmotic fragility and hemolysis of red blood cells were determined by using spectrophotometric technique. The results showed no statistically significant change in the number of burr cells, osmotic fragility, and hemolysis of red blood cells after an in vitro exposure to various concentrations of IRCMs when compared to the corresponding non-exposed control groups. These findings suggested that iohexol, iopamidol, and iodixanol did not exhibit any short-term effects on morphology, osmotic fragility, and hemolysis of red blood cells.

KEY WORDS: Iodinated radiographic contrast media; red blood cells; morphology; osmotic fragility; hemolysis

Introduction

Iodinated radiographic contrast media (IRCM) are widely used to visualize blood vessels in angiography, computed tomography and intervention radiology. IRCMs exhibit differences in their physicochemical properties and have either a higher or a lower effect on endothelial cells (Franke *et al.,* 2011; Franke *et al.*, 2007; Ramponi *et al.*, 2007) and on red blood cells as well (Kerl *et al.*, 2008; Reinhart *et al.*, 2005; Strickland *et al.*, 1992). Previously, authors have shown the effects of IRCMs on the morphology of red blood cells. Hardeman *et al.* (1991) showed that IRCMs (10%, 25%, 50%, and 75% v/v of iohexol and iopamidol) induced formation of echinocytes (Hardeman *et al.*, 1991). Jung *et al.* (2008) also showed that after incubation of red blood cells with iopromide and iodixanol in a concentration of 40% v/v, echinocytes were found (Jung *et al.*, 2008). In addition, Losco *et al.* (2001) reported that red blood cells from a normal volunteer exposed to 30% v/v of diatrizoate showed 100% of echinocytes formation (Losco *et al.*, 2001).

Moreover, there are several studies that have revealed the IRCMs-induced formation of echinocytes might involve plasma membranes of red blood cells. Franke *et al.* (2013) showed that IRCMs, iodixanol and iopromide, altered the spectrin/actin-network of the membrane cytoskeleton of red blood cells (Franke *et al.*, 2013). In addition, the spectrin/band3-network was likely involved in the morphological change of red blood cells after exposure to IRCMs (Franke *et al.*, 2014).

However, consequences of these effects are still being evaluated. In the present study, we focused on two biological endpoints that are known to be associated with a consequence of morphological change or plasma membrane disintegration of red blood cells. These biological endpoints are osmotic fragility and hemolysis. Osmotic

Correspondence address:
Montree Tungjai, Ph.D.
Department of Radiologic Technology,
Faculty of Associated Medical Sciences,
Chiang Mai University, Chiang Mai, Thailand, 50200
E-MAIL: mtungjai@gmail.com

fragility was considered as a biological endpoint based on whether abnormal morphology or volume of red blood cells can alter their osmotic fragility. It is known that osmotic fragility of red blood cells decreases, resulting in an increased risk of hemolysis incidence in red blood cells. The aim of this present study was to examine these IRCMs (i.e., iohexol, iopamidol, and iodixanol) in terms of their impact on morphology, osmotic fragility, and hemolysis of red blood cells.

Materials and methods

Chemicals

Three commercially available iodinated radiographic contrast media (IRCM) used were iohexol (omnipaque; GE Healthcare, China), iopamidol (iopamiro; Bracco, Italy), and iodixanol (visipaque; GE Healthcare, Ireland). These IRCMs are commonly used in diagnostic radiology.

Blood sample

Blood sample collections were performed under the approved guidelines set by the Institutional Committees on Research Involving Human Subjects and the approval of the Faculty of Associated Medical Sciences, Chiang Mai University. Peripheral blood samples (approximately 5 ml from each volunteer) were collected by venipuncture into heparinized syringes from 10 non-smoking healthy male volunteers (30–50 years old) who had no history of previous exposure to any clastogens.

Morphology

Blood was shortly treated with IRCMs (1, 5, 10, and 50 mgI/ml) at 37 °C for 5 minutes. Next, blood was dropped on the glass slide, and a blood smear was then performed. The blood smear slides were fixed with methanol and stained with Wright-Giemsa solution. The burr cells (echinocytes) were recorded under a light microscope. For consistency, the microscopic analysis was performed by a single individual (Benjamaporn Supawat). The slides were coded so that the analyst was not aware of the treatment and after the slides were scored the code was revealed.

Osmotic fragility (OF)

The osmotic fragility test was used to determine the degree of hemolysis. The authors determined the osmotic fragility of red blood cells using a spectrophotometric technique that was previously described (Tungjai *et al.*, 2018; Tungjai *et al.*, 2019). That previous method was performed with some slight modifications. Briefly, blood was shortly treated with IRCMs (1, 5, 10, and 50 mgI/ml) at 37 °C for 5 minutes. Next, 25 µl of blood was incubated in 1,000 µl of 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, and 0.05% sodium chloride solutions for 3 minutes at 37 °C. Afterward, samples were centrifuged at 7,000 rpm, for 1 minute. The release of hemoglobin into the supernatant was determined by spectrophotometer. The absorbance at 415 nm was plotted as a function of the concentration of sodium chloride solution. The osmotic fragility data were fitted by Boltzmann function and Gaussian function using the following equations:

Boltzmann equation:

$$y = \frac{A_1 - A_2}{1 + e^{\frac{(x - x_0)}{dx}}} + A_2$$

Where: A1 and A2 is the initial and final value, x0 is the center and dx is a constant.

Gaussian equation:

$$y = y_0 + \frac{A}{w\sqrt{\frac{\pi}{2}}} e^{-2\frac{(x - x_c)^2}{W^2}}$$

Where: y0 is the offset, A, xc and W is the area, center, and full width at half maximum, respectively.

The parameter values (xc, width, area, height) of the Gaussian curve were defined as follows: xc is the position of the peak on the X axis represents OF50 (the concentration of sodium chloride that could induce hemolysis of red blood cells by 50%). The maximum height of the peak indicates the maximum rate of hemolysis. The area of the curve is the rate of hemolysis. The width of peak is dispersion of hemolysis.

Hemolysis

The hemoglobin released from the cells was used as an indicator of red blood cells hemolysis. The authors determined the hemolysis of red blood cells using the spectrophotometric method previously described (Tungjai *et al.*, 2018). That previous method was performed with some modifications. Briefly, blood was shortly treated with IRCMs (1, 5, 10, and 50 mgI/ml) at 37 °C for 5 minutes. Next, 25 µl of blood was incubated in 725 µl of phosphate buffer saline (PBS), or in 725 µl of distilled water for 3 minutes at 37 °C. Next, samples were centrifuged at 7,000 rpm for 1 minute. The release of hemoglobin into the supernatant was determined by spectrophotometer. The absorbance (Abs) at 415 nm was used to calculate the percentage of hemolysis using the following equation: Percentage of hemolysis = (Abs (415 nm) in PBS / Abs (415 nm) in water) × 100. Where; Abs (415 nm) in PBS and Abs (415 nm) in water were the absorbance at 415 nm of the release of hemoglobin into PBS and water, respectively.

Statistical analysis

All experiments were performed three times (n=3). We expressed the results as mean ± standard error (SE). The Student's t-test was used independently to evaluate any statistical differences in the mean values between each test group and the corresponding control. A *p*-value of less than 0.05 was considered as statistically significant.

Results

Morphology

Figure 1 shows the percentage of burr cells in red blood cells after an *in vitro* exposure to various concentrations of IRCMs and in the corresponding non-exposed control groups. This data shows no changes occurring in the percentage of burr cells in iopamidol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.57–0.96), iohexol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.23–0.43), iodixanol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.06–0.61) compared to the corresponding non-exposed control groups.

Osmotic fragility (OF)

Table 1 shows the parameter values (xc, width, area, height) of the Gaussian curve that fit the osmotic fragility data of red blood cells after an *in vitro* exposure to various concentrations of IRCMs, and in the corresponding non-exposed control groups. Results show that no changes in the parameter values of the Gaussian curve took place that fit the osmotic fragility data of IRCMs-exposed red blood cells compared to the corresponding non-exposed control groups at all concentrations of iopamidol, iohexol, and iodixanol. This data indicates that IRCMs did not affect osmotic fragility of red blood cells.

Hemolysis

Figure 2 shows the percentage of hemolysis of red blood cells after an *in vitro* exposure to various concentrations of IRCMs, and in the corresponding non-exposed control groups. Similar to the percentages of burr cells in red blood cells and osmotic fragility, this data also indicated that no change in the percentage of hemolysis of iopamidol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.34–0.65), iohexol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.32–0.99), iodixanol-exposed red blood cells (Student's t-test, *p*-value ranging from 0.44–0.78) had occurred, compared to the corresponding non-exposed control groups.

Discussion

Previously, our studies suggested that IRCMs (i.e., iobitridol, iodixanol, iohexol, ioxaglate, and isovue) exhibited weak *in vitro* antioxidant properties. The antioxidant ability depended on the concentration of IRCMs (Tungjai *et al.*, 2018). Also, we examined IRCMs (i.e., iohexol, iopamidol, iobitridol, ioxaglate, and iodixanol) in terms of their cytotoxicity, effect on mitochondria membrane potential, and P-glycoprotein function in multidrug resistant K562/Dox cancer cells and corresponding

Table 1. The parameter values (xc, width, area, height) of the Gaussian curve that is fitting the osmotic fragility data of red blood cells after an *in vitro* exposure to various concentrations of IRCMs (iopamidol, iohexol, and iodixanol) and in the corresponding non-exposed control groups.

Lopamidol, mgI/ml	xc	width	area	height
0	0.44±0.02	0.18±0.03	0.40±0.03	2.19±0.32
1	0.47±0.01	0.14±0.03	0.42±0.04	3.22±0.56
5	0.46±0.02	0.12±0.03	0.41±0.03	3.47±0.50
10	0.45±0.01	0.13±0.02	0.43±0.05	2.76±0.35
50	0.46±0.02	0.12±0.02	0.32±0.03	2.85±0.59
Iohexol, mgI/ml				
0	0.45±0.02	0.14±0.02	0.40±0.02	3.42±0.82
1	0.41±0.02	0.15±0.03	0.42±0.04	3.33±0.81
5	0.42±0.03	0.16±0.02	0.41±0.04	2.51±0.47
10	0.43±0.02	0.16±0.03	0.42±0.04	3.23±0.85
50	0.41±0.03	0.13±0.02	0.33±0.03	2.03±0.26
Iodixanol, mgI/ml				
0	0.42±0.02	0.16±0.03	0.37±0.02	2.32±0.33
1	0.41±0.03	0.15±0.04	0.44±0.06	3.27±0.69
5	0.44±0.02	0.14±0.03	0.40±0.07	3.51±0.64
10	0.46±0.02	0.10±0.02	0.41±0.03	4.19±1.04
50	0.46±0.02	0.09±0.02	0.33±0.02	3.76±0.72

The value represents the mean±standard error of triplicate experiments. *$p<0.05$.

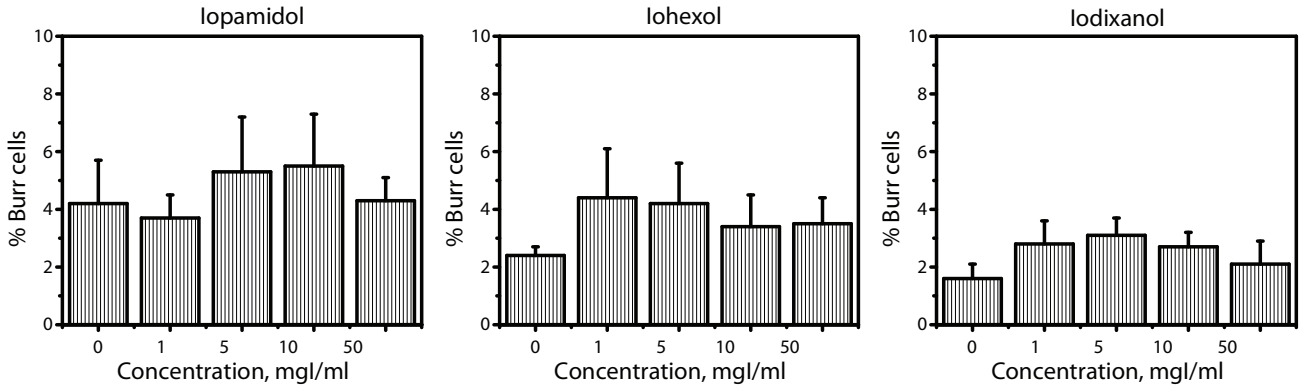

Figure 1. shows the percentage of burr cells in red blood cells after an in vitro exposure to various concentrations of IRCMs (iopamidol, iohexol, and iodixanol) and in the corresponding non-exposed control groups. The value represents the mean ± standard error of triplicate experiments. *$p<0.05$.

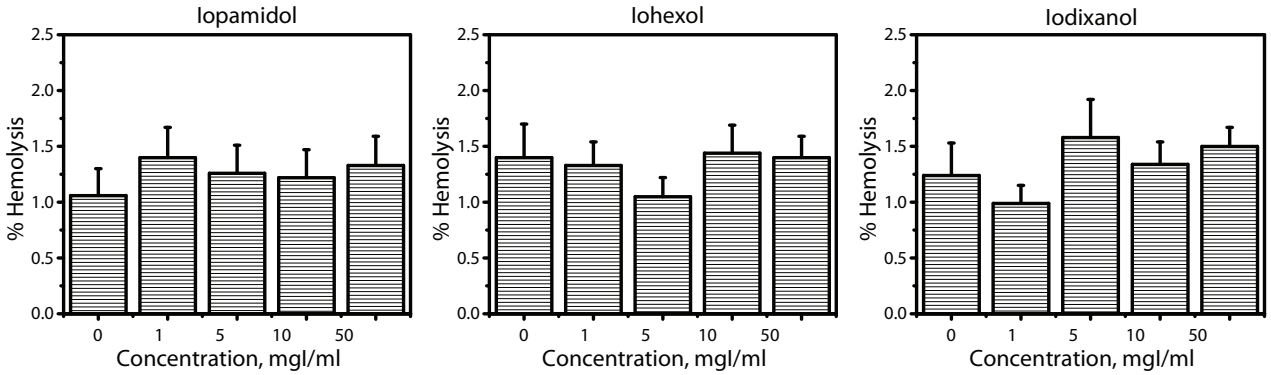

Figure 2. shows the percentage of hemolysis of red blood cells after an in vitro exposure to various concentrations of IRCMs (iopamidol, iohexol, and iodixanol) and in the corresponding non-exposed control groups. The value represents the mean ± standard error of triplicate experiments. *$p<0.05$.

sensitive cancer cells. The results showed that IRCMs exhibited cytotoxicity on multidrug resistant cancer cells and their corresponding sensitive cancer cells (Supawat *et al.*, 2019). Based on our two previous studies, those IRCMs have shown their impacts in a concentration dependent manner.

This more recent data demonstrated that there were no statistically significant changes in morphology, osmotic fragility, and hemolysis of red blood cells after *in vitro* exposure to IRCMs (iohexol, iopamidol, and iodixanol). Hence, *in vitro* data using these three biological endpoints suggested that IRCMs do not induce any detrimental effects on human red blood cells.

However, there is earlier reported evidence that the morphology of red blood cells can change after their suspension in IRCMs (Hardeman *et al.*, 1991; Jung *et al.*, 2008). In addition, morphology has been evaluated in order to study the effects of IRCMs (i.e.; iohexol, ioxaglate, and iodixanol) after an *in vitro* treatment of red blood cells to IRCMs (Galtung *et al.*, 2002). These results showed that red blood cells exposed to iohexol, ioxaglate, or iodixanol solutions shrank and then swelled. The authors indicated that there was change in morphology of red blood cells after an exposure to IRCMs. It should be noted that there was a difference in the experimental design between our present study and the study conducted by Galtung *et al.* (Galtung *et al.*, 2002) based on the type of incubation between red blood cells and IRCMs. The present study used whole blood that was directly incubated with IRCMs, while the study conducted by Galtung *et al.* (2002) (Galtung *et al.*, 2002) used whole blood that was exposed to ringer solutions containing IRCMs.

Moreover, another study also observed morphology using an *in vitro* study to determine the effects of IRCMs (i.e.; iodixanol and iopromide) after treatment of red blood cells (Franke *et al.*, 2014). These findings showed that echinocytes formation had occurred after red blood cells were exposed to iodixanol and iopromide. The authors also indicated that IRCMs induced rounded bubble-like protrusions from the cell membrane containing long bundles of actin fibers.

Likewise, there was a difference in the experimental design between the study conducted by Franke *et al.* (Franke *et al.*, 2014) and the present study. The study conducted by Franke *et al.* (Franke *et al.*, 2014) used plasma and red blood cells that was separated by centrifugation. Afterward, the red blood cells were then re-suspended in plasma/IRCMs mixtures. It is noteworthy that the differences in the experimental design i.e., type of incubation between cells and IRCMs, may have contributed to different findings regarding the effects of IRCMs on the cells.

Similar to the morphology study, these data showed no statistically significant changes in osmotic fragility of red blood cells after exposure to IRCMs. In contrast, another study indicated that IRCM diminished osmotic fragility of red blood cells after incubating for 30 minutes (Paul *et al.*, 1983). It is also important to note that the incubation time used in their studies was 5 times higher than those used in this present study. Thus, differences in incubation time (30 minutes vs. 5 minutes) may also contribute to the different findings regarding the effects of IRCM on osmotic fragility of red blood cells.

In way similar to the morphology and osmotic fragility study, these data also showed no statistically significant change in hemolysis of red blood cells after exposure to IRCMs. It is known that the abnormal morphology of red blood cells, such as in burr cells (echinocytes), can impact hemolysis of red blood cells (Gerk *et al.*, 2014). Therefore, it was not surprising that our results did not show IRCMs having any effect on hemolysis of red blood cells because these IRCMs did not change the morphology of red blood cells in any way. These results were consistent with the study that was conducted by Gerk *et al.* (Gerk *et al.*, 2014). These authors reported that two IRCMs, iodixanol and iopromide, slightly induced hemolysis of red blood cells (Gerk *et al.*, 2014).

Conclusion

These findings showed that there were no statistically significant changes in morphology, osmotic fragility, and

hemolysis of red blood cells after short-term exposure to iohexol, iopamidol, or iodixanol. Thus, *in vitro* results using these three biological endpoints suggests that IRCMs did not have any detrimental effects on human red blood cells.

Acknowledgments

We would like to thank the Department of Radiologic Technology and the Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University for use of their facilities and support.

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