# Influence of different radiographic contrast media on the echinocyte formation of human erythrocytes

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**Abstract.** Echinocyte formation is associated with a rigidification of the cells that may affect capillary perfusion and, consequently, the tissue oxygen supply. This study examines how many echinocytes appeared after the addition of radiographic contrast media (RCM) (Iodixanol320, Ioversol300, Iopamidol300, and Iomeprol400) compared to red blood cells in autologous plasma and in isotonic saline solution.

Isotonic saline solution, Iodixanol, Ioversol, Iopamidol and Iomeprol in concentrations of 10 vol%, 20 vol%, and 40 vol% were added to the plasma of seven healthy subjects. Subsequently, the erythrocytes were resuspended in these plasma/RCM mixtures, incubated for 5 minutes and then examined under the microscope.

The concentrations and the RCM in the mixture had a significant effect on the number of discocytes (factor concentration: $p < 0.0001$; factor RCM: $p < 0.0001$). The percentage of discocytes for all concentrations depended significantly on the RCM/plasma mixture (concentration × RCM: $p < 0.002$).

Of all RCM/plasma mixtures used, the Iodixanol/plasma mixture showed the most similar discocyte fraction compared to red blood cells in the autologous plasma. Importantly, while Iodixanol differed from all other RCMs, the other RCMs did not differ from one another with respect to the discocyte fraction.

## 1. Introduction

The conventional radiographic contrast media (RCM) used in radiographic studies are hyperosmolar. They can induce marked changes in shape, volume and resistance to deformation of red blood cells. In addition, a chemotoxic effect of RCMs was described [13], which was associated with an echinocyte formation and a rigidification of the erythrocytes [25–27, 29, 33, 35, 40], which can result in a decline of capillary perfusion [7, 8, 19, 20, 28, 37, 39]. The diminished convective oxygen transport in the capillaries and the limited oxygen-uptake/delivery of the echinocytes can result in a decrease of the tissue oxygen partial pressure [16]. Measurements of the oxygen partial pressure in the beating cardiac muscle of pigs after bolus administration of various RCMs into the left coronary artery revealed striking differences [20,

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28]: The decrease of the myocardial oxygen partial pressure was much stronger, e.g. after Iomeprol than after Iodixanol [28].

Reports on echinocyte formation after exposure of erythrocytes to RCM have already been presented on several occasions [5, 6, 18, 21, 27, 42]. In these studies, different concentrations (between 10% and 50% by volume RCM) and differing procedures (various anticoagulants and analytical procedures) were used so that a comparison among these studies is possible only to a limited extent. For this reason, the effects of various RCMs on erythrocyte morphology were examined under well standardized experimental conditions and evaluation procedures.

## 2. Materials and methods

Aim of the study was, to analyze, whether RCM influenced the morphology of erythrocytes in comparison to NaCl and autologous plasma. The study was performed in accordance with the ethical guidelines of the journal [2]. Four radiographic contrast media approved for intraarterial application were examined (see Table 1).

### 2.1. Blood collection

Venous blood (20 mL) was collected in a standardized manner from the cubital veins of $n = 6$ healthy adults according to the Nordkem workshop criteria [1] without using a tourniquet and anticoagulated with EDTA. Sterile disposable large caliber cannules were used for blood sampling [9]. The samples were stored in sealed polystyrene tubes. None of the donors were taking medication before or during the study. Donors were informed and gave written consent.

### 2.2. Sample processing

Plasma and an erythrocyte concentrate with a hematocrit of 98% were obtained by centrifugation. In the next step, the plasma/RCM mixtures required for resuspending the erythrocytes were prepared. Isotonic NaCl, or the RCMs were added to the plasma in the concentrations of 10%, 20% or 40%, which were considered reasonable concentrations to simulate the bolus injection phase in arteriography as required for successful imaging [24]. Then, the red blood cells were resuspended in these mixtures and incubated for 5 minutes at 37°C.

Table 1
Radiographic contrast media

|  | Concentration of iodine (mg/ml) | Osmolality mOsmol/kg water |
| --- | --- | --- |
| (1) Iodixanol (Visipaque™) | 320 | 290 |
| (2) Ioversol (Optiray™) | 300 | 645 |
| (3) Iopamidol (Solutrast™) | 300 | 616 |
| (5) Iomeprol (Imeron™) | 400 | 726 |

*2.3. Microscopy*

To count the number of echinocytes in a microscope, a 10 µl suspension drop of each mixture was placed on Menzel glass slides, which had been washed before in a mixture of alcohol (20% v/v) and acetic acid (2% v/v) in bidistilled water. In a transmission light microscope (Zeiss Axiom Imager.Z2 m, Camera: Zeiss AxioCam HRc, software: AxioVision Rel.4.8.2), the percentage of discocytes, echinocytes (stages I to III according to Bessis [11, 12]) or of other cell shapes (acantocytes, drepanocytes (synonymous to helmet cells or blister cells), keratocytes, cnizocytes, elliptocytes, leptocytes, spherocytes, stomatocytes, torocytes (also known as codocytes or target cells) among the total number of erythrocytes was determined.

The cells were counted using a 40-fold primary magnification. The observer meandered through as many fields of vision as were necessary to characterize a total of 100 cells.

*2.4. Statistics*

All samples were described using mean value and standard deviation. The test statistics were performed using two-factorial analysis of variance (factor 1: medium, factor 2: concentration). The test variable was the percentage of normal erythrocytes (discocytes). The null hypothesis was: The fraction of discocytes after incubation of the erythrocytes in the various media at 3 concentrations and at 37°C for 5 minutes exhibits no differences for the 3 concentrations. The null hypothesis was rejected with a probability α of less than 0.05.

## 3. Results

Four male and three female apparently healthy subjects were stratified and 6 of them were included (in accordance with stringent inclusion criteria [22]). The average age was $35.7 \pm 8.6$ years, the average weight $70.4 \pm 10.2$ kg, and the average height $176.3 \pm 4.2$ cm. Cardiovascular risk factors (diabetes mellitus, arterial hypertension, hyperlipidemia, smoking) were excluded. None of the subjects were taking medication before or during the study.

*3.1. Confirmatory parameter influence of the four radiographic contrast media on the percentage of discocytes*

The type of RCM (see Table 2) added to the plasma had a significant influence on the number of discocytes ($p < 0.001$). The second factor, the concentration of RCM in plasma, too, had a significant influence on the percentage of discocytes ($p < 0.001$).

The most important result revealed that the percentage of discocytes, for all RCM concentrations chosen, significantly depended on the type of medium the erythrocytes were incubated in ($p = 0.002$).

Figure 1 gives an overview of all results. In a comparison of all the RCMs examined in this study, only the fraction of discocytes in the Iodixanol/plasma mixture was similar to and nearly as low as the fractions either in autologous plasma or in the Na/Cl plasma mixture. The biggest differences appeared at the higher RCM concentrations which are used in clinical reality when RCM are applied in coronary arteries as a bolus injection in order to gain an iodine delivery rate as high as possible and an excellent contrast of coronary blood vessels.

Table 2

2-Factor ANOVA analysis of the fraction of discocytes in autologous plasma, in a NaCl/plasma mixture, in a Iodixanol/ plasma mixture, in a Iopamidol/plasma mixture, in a Ioversol/plasma mixture and in a Iomeprol/plasma mixture, at RCM concentrations of 10% v/v, 20% v/v or 40% v/v

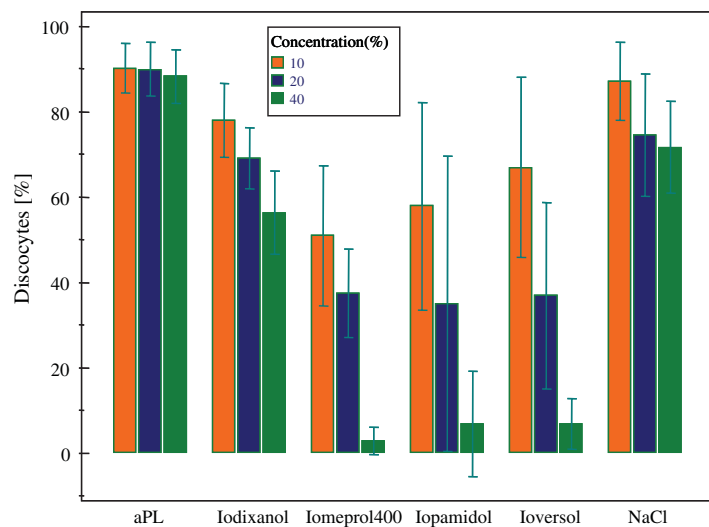| Source of variation | Sum of squares | DF | Mean square | $f$ | $p$ |
|---|---|---|---|---|---|
| RCM | 58,822.139 | 5 | 11,764.428 | 43.75 | <0.001 |
| Concentration | 19,700.085 | 2 | 9,850.043 | 36.63 | <0.001 |
| 2-Factor interactions | 8,533.288 | 10 | 853.329 | 3.17 | 0.002 |
| Residual | 24,200.085 | 90 | 268.890 | | |



Fig. 1. Percentage of discocytes in the four RCM/plasma mixtures examined (Iodixanol320, Iomeprol400, Iopamidol300 and Ioversol300) in comparison to whole blood (aPL) and to isotonic saline (NaCl). (Mean value ± standard deviation).

With respect to the percentage of discocytes, Iodixanol clearly and significantly differed from the other RCMs tested, whereas the other three RCMS statistically did not differ from one another regarding the percentage of discocytes.

Table 3 shows the *p*-values of the influence of the various RCMs on the percentage of discocytes.

### 3.2. Echinocyte formation after the addition of 3 different RCM concentrations (10% v/v, 20% v/v, 40% v/v)

Table 4 shows the percentages of all the echinocytes present and of echinocytes in the three subgroupes according to Bessis (each of them related to the total number of echinocytes).

The missing percentage fraction (adding discocytes (Table 2) and echinocytes we do not arrive at 100%) accounts for specially shaped erythrocytes like akanthocytes, drepanocytes (also called Helmet cells or bliester cells), keratocytes, knizocytes, elliptocytes, leptocytes, spherocytes, sickle cells, stomatocytes, torocytes (also called codocytes or target cells) and others.

Table 3

Influence of the four RCMs tested (Iodixanol320, Iomeprol400, Iopamidol300, and Ioversol300) on the percentage of discocytes, table of significance

| | | | |
|---|---|---|---|
| Iodixanol | – | Iopamidol | $p < 0.0001$ |
| Iodixanol | – | Ioversol | $p < 0.0001$ |
| Iodixanol | – | Iomeprol | $p < 0.0001$ |
| Iopamidol | – | Ioversol | $p = 0.515$ |
| Iopamidol | – | Iomeprol | $p = 0.9545$ |
| Iomeprol | – | Ioversol | $p = 0.624$ |

Table 4

Fraction of echinocytes in percent of all cells assessed in the analysed areas after incubation in the four RCMs (Iodixanol320, Iomeprol400, Iopamidol300, and Ioversol300) in comparison to cells incubated in whole blood or in isotonic saline. (Mean value ± standard deviation)

| | Total number of echinocytes and subgroups according to Bessis | | | |
|---|---|---|---|---|
| | Total [%] | I [%] | II [%] | III [%] |
| Whole blood-10% | 9.33 ± 6.26 | 97.2 ± 6.4 | 2.78 ± 6.8 | 0.0 ± 0.0 |
| Whole blood-20% | 9.51 ± 6.38 | 97.2 ± 6.4 | 2.78 ± 6.8 | 0.0 ± 0.0 |
| Whole blood-40% | 9.80 ± 6.56 | 97.2 ± 6.4 | 2.78 ± 6.8 | 0.0 ± 0.0 |
| Saline-10% | 11.4 ± 8.77 | 100 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Saline-20% | 23.4 ± 13.9 | 98.1 ± 3.3 | 1.93 ± 3.29 | 0.0 ± 0.0 |
| Saline-40% | 24.6 ± 10.9 | 91.7 ± 6.64 | 8.35 ± 6.64 | 0.0 ± 0.0 |
| Iodixanol-10% | 18.29 ± 7.62 | 95.0 ± 7.99 | 4.99 ± 7.99 | 0.0 ± 0.0 |
| Iodixanol-20% | 28.2 ± 8.77 | 94.2 ± 12.7 | 5.82 ± 12.7 | 0.0 ± 0.0 |
| Iodixanol-40% | 39.9 ± 11.9 | 85.5 ± 16.4 | 14.0 ± 15.4 | 0.26 ± 0.64 |
| Ioversol-10% | 32.4 ± 23.5 | 89.1 ± 10.1 | 10.6 ± 9.61 | 0.33 ± 0.81 |
| Ioversol-20% | 61.8 ± 24.2 | 76.8 ± 13.2 | 20.2 ± 11.6 | 2.97 ± 2.53 |
| Ioversol-40% | 92.8 ± 6.98 | 47.3 ± 23.7 | 38.2 ± 11.8 | 14.5 ± 12.9 |
| Iopamidol-10% | 40.9 ± 27.5 | 89.6 ± 11.5 | 10.1 ± 11.1 | 0.23 ± 0.56 |
| Iopamidol-20% | 64.7 ± 38.2 | 69.1 ± 32.1 | 26.1 ± 25.9 | 4.80 ± 6.48 |
| Iopamidol-40% | 92.8 ± 13.5 | 41.9 ± 23.1 | 41.2 ± 10.7 | 16.9 ± 20.6 |
| Iomeprol-10% | 30.3 ± 14.7 | 92.5 ± 7.92 | 6.85 ± 6.59 | 0.69 ± 1.70 |
| Iomeprol-20% | 37.7 ± 6.74 | 79.3 ± 8.05 | 20.7 ± 8.05 | 0.0 ± 0.0 |
| Iomeprol-40% | 62.2 ± 11.3 | 39.9 ± 31.1 | 51.1 ± 25.6 | 9.01 ± 6.92 |

The incidence of echinocyte formation differed markedly after the addition of the four RCMs to the incubation medium ($p < 0.001$; see ANOVA Table 5).

The differences between erythrocytes incubated in the single RCM/plasma mixtures or in the control (in autologous plasma) or in the NaCl/plasma mixture are shown in Table 6.

Table 5

ANOVA table: incidence of echinocyte formation differed markedly after the addition of the four RCMs to the incubation medium

| Source of variation | Sum of squares | DF | Mean square |
|---|---|---|---|
| Between groups (influence factor) | 54,327.8841 | 5 | 10,865.5768 |
| Within groups (other fluctuations) | 47,355.5820 | 102 | 464.2704 |
| Total | 101,683.4661 | 107 | |
| $F$-ratio | | | 23.404 |
| Significance level | | | $p < 0.001$ |

Table 6

Table of significance for the influence of the six incubation media on the formation of echinocytes

| Factor | n | Mean | Different ($p < 0.05$) from factor nr |
|---|---|---|---|
| (1) aPL | 18 | 6.0556 | (3) (4) (5) |
| (2) Iodixanol | 18 | 21.7222 | (3) (4) (5) |
| (3) Iomeprol400 | 18 | 43.3889 | (1) (2) (4) (5) (6) |
| (4) Iopamidol | 18 | 66.1607 | (1) (2) (3) (6) |
| (5) Ioversol | 18 | 62.3253 | (1) (2) (3) (6) |
| (6) NaCl | 18 | 19.8015 | (3) (4) (5) |

Table 7

Comparisons for factor: concentration of autologuous plasma

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
|---|---|---|---|---|---|
| 40,000 vs. 20,000 | 1.50 | 0.16 | 0.873 | 0.017 | No |
| 40,000 vs. 10,000 | 1.33 | 0.14 | 0.887 | 0.025 | No |
| 10,000 vs. 20,000 | 0.17 | 0.02 | 0.986 | 0.050 | No |

After the addition of Iodixanol considerably less echinocytes were formed than after the addition of the other three RCMs (Iomeprol, Iopamidol und Ioversol) with each $p$-value <0.05. There was no statistical difference in echinocyte formation after erythrocyte incubation either in Iodixanol, or in autologous plasma or in the NaCl/plasma mixture. Figure 2 demonstrates these differences.

Table 2 revealed that the concentration of the medium added to the medium/plasma mixture (for all of the 5 media) had an influence on the fraction of discocytes. Now, it was analysed whether the concentration of the RCM added to the mixture also influenced the number of echinocytes.

The substitution of autologous plasma by autologous plasma did not influence the rate of echinocyte formation (see Table 7). Nor did the substitution of autologous plasma by isotonic NaCl solution influence the echinocyte formation (see Table 8).

Neither did the addition of Iodixanol to the plasma influence the rate of echinocyte formation (see Table 9).

On the contrary, the rate of echinocyte formation (see Tables 10, 11 and 12) was markedly enhanced after the incubation of erythrocytes in RCM/plasma mixtures when the other three RCM were contained.
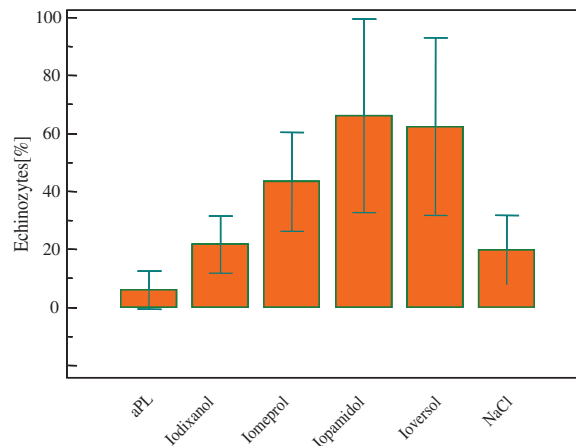
Fig. 2. Mean total numbers of echinocytes in % and averaged over the three concentrations after the incubation of erythrocytes in three different concentrations (10% v/v, 20% v/v, and 40% v/v) of autologous plasma (aPL), of a NaCl/plasma-, a Iodixanol/plasma-, a Iomeprol/plasma-, a Iopamidol/plasma- and a Ioversol/plasma-mixture.

Table 8

Comparisons for factor: concentration of NaCl

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
|---|---|---|---|---|---|
| 40,000 vs. 10,000 | 13.23 | 1.41 | 0.161 | 0.017 | No |
| 20,000 vs. 10,000 | 12.12 | 1.29 | 0.199 | 0.025 | No |
| 40,000 vs. 20,000 | 1.11 | 0.12 | 0.906 | 0.050 | No |

Table 9

Comparisons for factor: concentration of Iodixanol

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
|---|---|---|---|---|---|
| 40,000 vs. 10,000 | 15.00 | 1.60 | 0.112 | 0.017 | No |
| 40,000 vs. 20,000 | 8.33 | 0.89 | 0.375 | 0.025 | No |
| 20,000 vs. 10,000 | 6.67 | 0.71 | 0.478 | 0.050 | No |

Table 10

Comparisons for factor: concentration of Iopamidol

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
|---|---|---|---|---|---|
| 40,000 vs. 10,000 | 51.93 | 5.55 | <0.001 | 0.017 | Yes |
| 40,000 vs. 20,000 | 28.11 | 3.01 | 0.003 | 0.025 | Yes |
| 20,000 vs. 10,000 | 23.82 | 2.55 | 0.013 | 0.050 | Yes |

With the exemption of Iodixanol, all of the other RCM examined here displayed a strong influence of concentration on the rate of formation ($p < 0.01$ each), with increasing RCM concentrations more and more echinocytes appeared. In the following figures examples are shown of erythrocytes in autologous plasma (see Fig. 3), in a NaCl/plasma mixture (see Fig. 4) and in RCM/plasma mixtures with any of the four different RCM (see Figs. 5, 6, 7, 8).

Table 11
Comparisons for factor: Concentration of Ioversol

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
| --- | --- | --- | --- | --- | --- |
| 40,000 vs. 10,000 | 60.40 | 6.46 | <0.00 | 0.017 | Yes |
| 40,000 vs. 20,000 | 30.94 | 3.31 | 0.001 | 0.025 | Yes |
| 20,000 vs. 10,000 | 29.47 | 3.15 | 0.002 | 0.050 | Yes |

Table 12
Comparisons for factor: Concentration of Iomeprol

| Comparison | Diff of means | $t$ | Unadjusted $p$ | Critical level | Significant? |
| --- | --- | --- | --- | --- | --- |
| 40,000 vs. 10,000 | 31.83 | 3.40 | <0.001 | 0.017 | Yes |
| 40,000 vs. 20,000 | 24.50 | 2.62 | 0.010 | 0.025 | Yes |
| 20,000 vs. 10,000 | 7.33 | 0.78 | 0.435 | 0.050 | No |
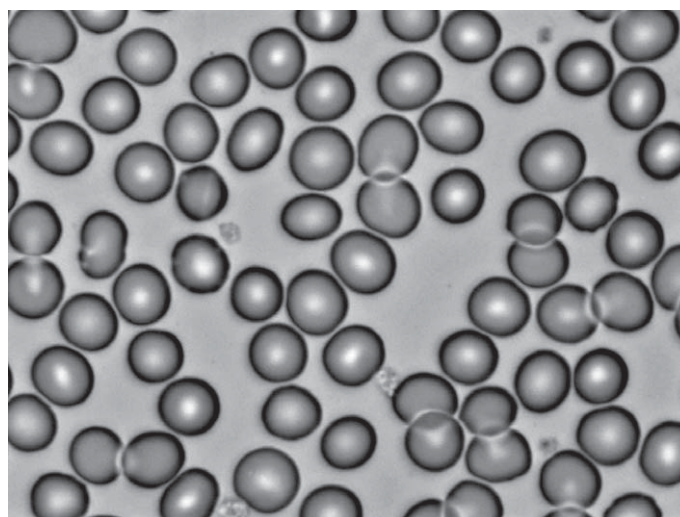


Fig. 3. Erythrocytes in autologous plasma.

## 4. Discussion

In EDTA-anticoagulated blood – after separation of red blood cells from plasma by centrifugation and re-incubation of the cells in autologous plasma – only a few echinocytes (less than 5%) in the smear could be detected (without washing, staining or fixation of the cells). Even the addition of an isotonic saline solution resulted in an increase of the number of echinocytes: from $11.4 \pm 8.77\%$ after addition of 10% saline (by volume) increasing to $24.6 \pm 10.9\%$ after addition of 40% saline (by volume/showing first echinocytes type II according to Bessis). This is in line with a former study [21] but also with studies showing that saline solution influenced the plasmatic coagulation [23, 34]. Evidently, isotonic saline
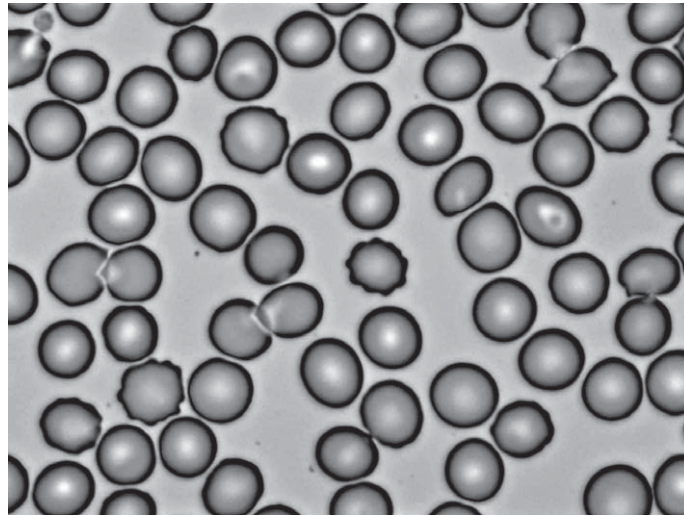
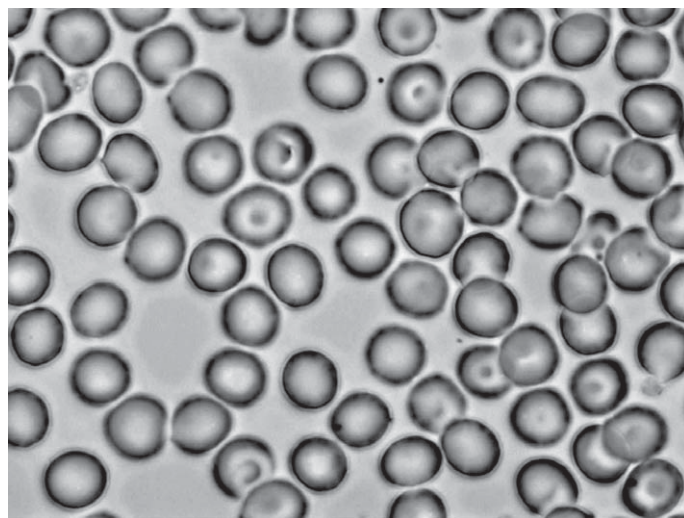Fig. 4. Erythrocytes in autologous plasma containing 40% NaCl-solution.



Fig. 5. Erythrocytes in autologous plasma containing 40% Iodixanol320.

solutions not only can interfere with the coagulation system but also with the blood cells. As mechanisms dilutional effects were discussed as well as changes in the electrolyte concentrations.

Of the RCMs examined here, only the addition of Iodixanol resulted in discocyte numbers comparable to RBCs incubated in isotonic saline solution/plasma mixture. In addition, fully developed echinocytes of grade III were not detected. The other RCMs, especially at higher concentrations, resulted in clearly increased echinocyte fractions. Iopamidol and Ioversol exhibited extremely high fractions of echinocytes of 92.8% after addition of 40% RCM (by volume). The result that Iopamidol induced an echinocyte formation of nearly all cells was published by Hardeman et al. and Aspelin et al [6, 17, 18] already.
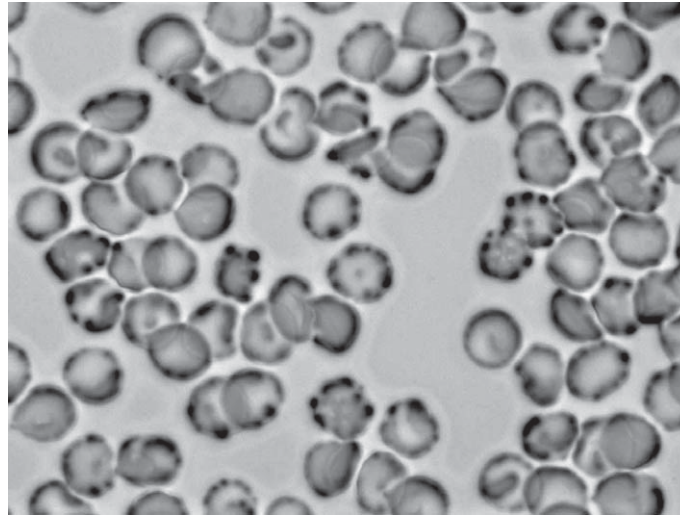
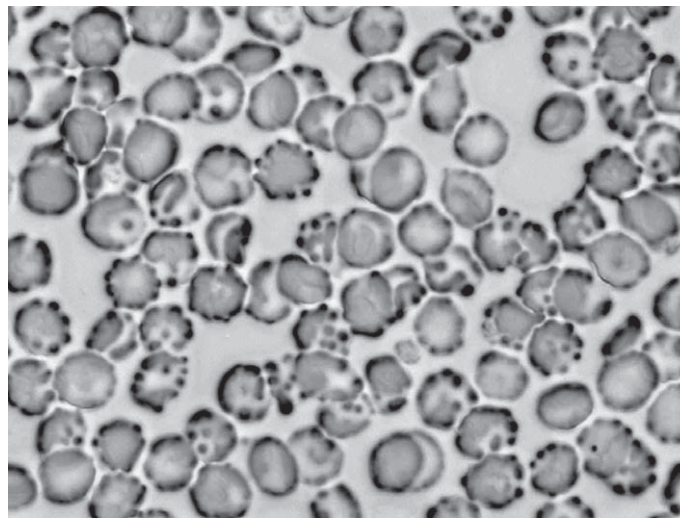Fig. 6. Erythrocytes in autologous plasma containing 40% Ioversol300.



Fig. 7. Erythrocytes in autologous plasma containing 40% Iopamidol300.

Under physiological conditions, a normal human erythrocyte assumes a biconcave discoid (discocyte) shape of 7.5 μm in diameter [32]. A variety of agents were shown to systematically modify the cell shape [10, 12, 30, 32, 38, 41]. Among these, anionic amphipaths as well as high salt concentrations, high pH and high osmolality values, ATP depletion, cholesterol enrichment and proximity to a glass surface induced strong increases in the number of echinocytes, characterized by convex rounded protrusions or spicules. Increased osmolality in blood, in particular, resulted in echinocyte formation, as was repeatedly shown, after the application of RCMs [4, 5, 18, 26].
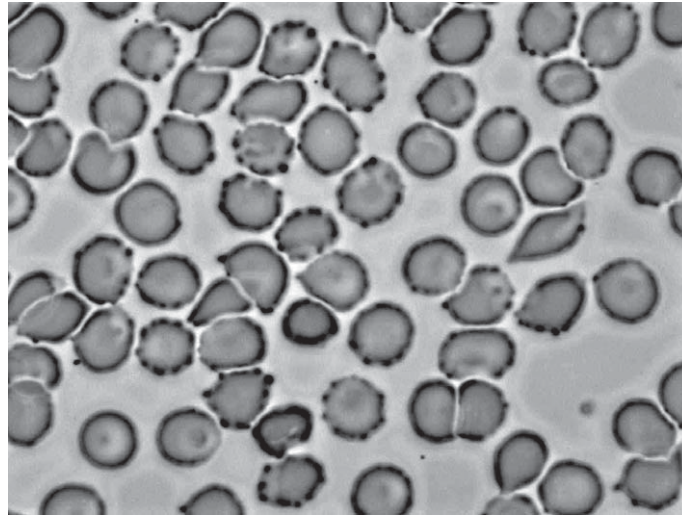
Fig. 8. Erythrocytes in autologous plasma containing 40% Iomeprol400.

In this study, the contrast medium with the highest osmolality (Iomeprol400) at a concentration of 40% (v/v) caused an echinocyte fraction of $62.2 \pm 11.3\%$, while the RCMs Iopamidol and Ioversol with lower osmolalities induced significantly higher echinocyte fractions showing that, evidently, not only the osmolality is a moving factor in the morphological changes of the erythrocyte membrane.

Several authors [3, 17, 21, 25, 38] confounded that factors other than osmolality seem to contribute to the echinocyte formation. A strong argument was supplied when contrast media, brought to isotonicity by dilution, still caused echinocyte formation [3, 6]. The precise interactions between the RCM molecules and the erythrocyte membrane on the molecular level are currently unknown. As causative mechanisms the apposition to or the embedding into the erythrocyte membrane of RCM molecules were discussed because the intercalation of molecules into the inner leaflet of the lipid bilayer of cell membranes could expand the outer leaflet relative to the inner one and thus induce the formation of echinocytes (bilayer-couple model [31, 36]). However, very recently it could be shown, that even at the highest convenient magnification (1 : 40,000) it was impossible to detect RBC membrane associated iodine after RBC incubation in RCM *in vitro* [15]. Neither in the birds view on the samples nor looking from the side on the freeze fractured samples carrying the RBC, it was possible to detect either the signal cohorts typical of iodine in the sum spectra or the main L$\alpha$1-peak in trace analysis.

## 5. Conclusion

Iodixanol added to the plasma of apparently healthy subjects induced a comparable echinocyte formation like the addition of isotonic saline solution which in this respect differed significantly from the other radiographic contrast media studied. This result is in good agreement with a previous study of our group and with results of Losco et al, who showed that only after the application of Iodixanol [27] there was no negative effect on the filterability of erythrocytes from sickle cell donors.

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