Laboratory Investigations

Super Absorbent Polymer Microspheres Prepared with Hypertonic Saline to Reduce Microsphere Expansion

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Key word
TACE, drug-eluting microsphere, Cisplatin

Short title

Super Absorbent Polymer Microsphere*s* with Reduced Expansion

Abstract

Purpose:

To analyze size changes of super absorbent polymer (SAP) microspheres with the reduced expansion technique, and to evaluate pharmacological advantages of transarterial chemoembolization using cisplatin-loaded SAP microspheres with the reduced expansion technique.

Materials and Methods:

In an in vitro study (Editor Q2), diluted contrast materials containing different concentrations of sodium ions were examined to expand SAP microspheres and determined the reduced expansion technique. Size distributions of cisplatin-loaded SAP microspheres were analyzed. In an in vivo study (Editor Q2), TACE was performed using cisplatin-loaded SAP microspheres with the reduced expansion and control techniques in 18 VX2 rabbits (Editor Q2).

Results:

The degree of expansion was reduced to the greatest extent by using a mixture of nonionic contrast material and 10 % NaCl at a 4:1 ratio (NaCl 2 w/v%). The mean diameter of the reduced expansion of cisplatin-loaded SAP microspheres was 188.4 μm, while that of the control expansion was 404.9 μm. The plasma concentrations of the control group at 5 minutes after TACE were significantly higher than those of the reduced expansion group (2.19 ± 0.77 *vs* 0.75 ± 0.08 μg/mL, $P = .01$). The tumor platinum concentrations of the reduced expansion group at 1 hour were significantly higher than those of the control group (10.76 ± 2.57 *vs* 1.57 ± 0.14 μg/g, $P = .044$).

Conclusion:

The expanding level of SAP microspheres can be reduced by using hypertonic saline. Cisplatin-loaded SAP microspheres with the reduced expansion technique have the advantages of achieving higher cisplatin tissue concentration in TACE for liver tumors.

Introduction

Drug-eluting microspheres have been developed and often used in transarterial chemoembolization (TACE) for liver tumors [1-5]. Super absorbent polymer (SAP) microspheres (HepaSphere; Merit Medical, South Jordan, Utah, USA) are the only microspheres that can load cisplatin by the unique characteristic of mechanical absorption without an ion-exchange process [6].

SAP microspheres are a hydrophilic copolymer with the property of expansion by the absorption of water in the microspheres [7, 8]. Regarding the mechanism of the absorption, when SAP microspheres contact with water, sodium ions ionize away from the carboxyl groups into the microspheres and the ion concentration difference occurs between the inside and the outside of the hydrophilic copolymer of SAP microspheres. Consequently, the water is absorbed into the hydrophilic copolymer and the microspheres expand.

It is known that SAP microspheres mixed with nonionic contrast medium expand approximately four times larger than original sizes in the dry stage [9]. Theoretically, in sodium ions containing water, the hydrophilic copolymer could absorb less water due to little ion concentration difference between the inside and the outside of the hydrophilic copolymer. As a result, the expansion level (R2, Q21) could be reduced compared with the non-sodium ionic water.

The smaller size microspheres should be ideal for tumors with fine feeding arteries allowing deep penetration into the tumor [10-12]. However, it remains a dilemma whether the amount of cisplatin-loaded in microspheres could be reduced in the less expanded microspheres.

Based on the above background, firstly an in vitro study was conducted to compare the level of expansion of SAP microspheres in various concentrations of sodium ions containing solvents, and the size distribution of cisplatin-loaded SAP microspheres produced by the reduced expansion technique was examined. Secondly, an in vivo study of TACE using a rabbit VX2 liver tumor model was conducted to compare the pharmacokinetics findings of cisplatin-loaded SAP microspheres between the reduced and control expansion techniques.

Materials and Methods

In Vitro Study of Expanding Levels of SAP Microspheres

In the first part of the in vitro study, the following four types of diluted or non-diluted contrast materials containing different concentrations of sodium ions (Fluid A, B, C, and D) were prepared at room temperature to compare the level of expansion of SAP microspheres. The total volume of each fluid was 10 mL. Fluid A was composed of 5mL of Iohexol 350 mg I/mL (Omunipaque; Daiichi Sankyo, Tokyo, Japan) and 5 mL of 10 % NaCl (NaCl 5 w/v%), Fluid B was 8 mL of Iohexol and 2 mL of 10 % NaCl (NaCl 2 w/v%), Fluid C was 9 mL of Iohexol and 1 mL of 10 % NaCl (NaCl 1 w/v%), and Fluid D was 10 mL of Iohexol (NaCl 0 w/v%). SAP microspheres with a dry size of 50-100 μm were expanded in each fluid for 15 minutes. Around 100 expanded microspheres were randomly sampled and the mean diameters and the size distribution were evaluated using a digital microscope (VHX-1000; Keyence, Osaka, Japan). Then, the fluid which mostly suppressed swelling of SAP microspheres was defined as the reduced expansion techniques.

In the second part of the in vitro study, cisplatin-loaded SAP microspheres created using the above-reduced expansion techniques were evaluated. A fine-powder formulation of cisplatin at a dose of 50 mg (IA-Call; Nippon Kayaku, Tokyo, Japan) was dissolved in 10 mL of Iohexol 350 mg I/mL at about 40℃. The cisplatin solution was mixed with 10% NaCl using the ratio of the reduced expansion technique according to the result of the first part of the in vitro study. Then, SAP microspheres with a dry size of 50-100 μm were expanded using this mixture, which was defined as the reduced expansion of cisplatin-loaded SAP microspheres. In addition, the cisplatin solution was mixed with physiological saline (0.9% NaCl) instead of 10 % NaCl using the same ratio and SAP microspheres were expanded, which was defined as the control expansion of cisplatin-loaded SAP microspheres. The size distributions of both cisplatin-loaded SAP microspheres were analyzed and microscopic examinations were conducted.

In Vivo Animal Study

The study protocol was approved by the Animal Experimentation Committee of our institution, and all experiments were performed in accordance with the Animal Care Guidelines of our institution. New Zealand white rabbits weighing 2.90–4.15 kg (mean 3.44 kg) were purchased from Japan SLC Inc. (Hamamatsu, Japan). VX2 tumors were implanted in the left lobe of the livers under laparotomy two weeks prior to TACE.

Eighteen rabbits with VX2 liver tumors were divided into two groups: the reduced expansion (n=9) group and the control (n=9) group. The reduced expansion and the control expansion of cisplatin-loaded SAP microspheres were prepared according to the results of the second part of the in vitro study.

Cisplatin-loaded SAP microspheres were injected via a 1.7 Fr microcatheter into

the left hepatic artery under fluoroscopic guidance in an angiography suite (Surginix; Toshiba, Otahara, Japan). The endpoint of the injection in both groups was a stasis of the left hepatic arterial flow.

The plasma concentrations of platinum were measured with an atomic absorption spectrometry (AAS) before the treatment and 5 minutes, 0.5, 1, 2, 24, and 72 hours after TACE. Plasma ultrafiltrate or diluted plasma was directly introduced to the AAS instrument. For tissues, samples were wet-ashed by nitric acid and the platinum was extracted as diethyldithiocarbamate-platinum complex using a chloroform. The extracted platinum complex was applied to the AAS. In the plasma ultrafiltrate, limit of detection (LOD) and lower limit of quantification (LOQ) were 0.002 and 0.01 μg/mL, respectively. In the non-filtered plasma, LOD and LOQ were 0.01 and 0.05 μg/mL, respectively. In the tissues, LOD and LOQ were 0.017 and 0.033 μg/g tissue, respectively.(R2, Q7)

The serum level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured before the treatment and 1, 24 and 72 hours after TACE. All rabbits were euthanized with an overdose of pentobarbital at 1 hour (n=3), 24 hours (n=3), and 72 hours (n=3) after TACE in both groups.

The liver tumors were cut in half along the midline and half were immediately frozen to measure the tumor platinum concentration. The remaining half was embedded in paraffin and stained with hematoxylin and eosin for histopathological evaluation. Tumor necrosis rates at 1, 24 and 72 hours were calculated as a percentage of the tumor necrosis area for each slice by an independent pathologist blinded to the treatments. The tumor necrosis ratio was estimated by visual calculation. (R1, Q5)

Statistical Analysis

All in vivo study data were provided as arithmetic mean ± SD. Pairwise comparisons of these values between the reduced expansion and the control groups were performed with Student's t-test. Values of $p < .05$ were considered significant. These analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, Ill. USA). The area under the concentration-time curve (AUC) calculations of the plasma concentration of platinum were performed using Phoenix WinNonlin (Certara G.K.; Princeton, NJ, USA).

Results

In Vitro Diameter Change of SAP Microspheres

Mean (min - max) diameter of SAP microspheres in dry-state was 79.9 (47.7 – 106.1) μm, and in the Fluids A, B, C and D were 211.0 (133.8 – 313.2) μm, 198.3 (130.2 – 280.4) μm, 230.6 (135.3 – 344.8) μm, 523.1 (342.7 – 653.5) μm, respectively. The cumulative size distributions are (R2, Q21) shown in Fig 1. The swelling of SAP microspheres was mostly suppressed in Fluid B. Therefore, the mixture using 8 mL of non-ionic contrast material and 2 mL of 10 % NaCl (NaCl 2 w/v%) was determined as the reduced expansion technique.

According to the above results, the reduced expansion of cisplatin-loaded SAP microspheres was produced using a cisplatin powder solution dissolved by 8 mL of Iohexol combined with 2 mL of 10% NaCl. The control expansion was produced using a cisplatin powder solution dissolved by 8 mL of Iohexol combined with 2 mL of saline.(R2, Q5) The mean (min - max) diameter of the reduced expansion of cisplatin-loaded SAP microspheres was 188.4 (82.2 – 298.5) μm, while that of the control expansion was 404.9 (220.6 – 566.9) μm (Table 1) (R2, Q9). The mean size of the reduced expansion of cisplatin-loaded SAP microspheres was 2.4 times larger than that of the dry size of SAP

microspheres, while that of the control expansion was 5.1 times larger than the dry size. The cumulative size distributions were shown in Fig 2. The microscopic findings showed calibrated and spherical shapes of cisplatin-loaded SAP microspheres, which were consistent with the results of the measurement of the sizes (Fig 3).

In Vivo Pharmacological and Histological Findings

The mean administered doses of cisplatin were 1.01 ± 0.19 mg/kg in the reduced expansion group and 0.90 ± 0.17 mg/kg in the control group (P = .26).

The total platinum concentrations in plasma peaked after 5 minutes in both groups. The maximum concentrations (C-max) were 2.19 ± 0.77 μg/mL in the reduced expansion group and 0.75 ± 0.08 μg/mL in the control group (P = .01). The total plasma platinum concentrations at 72 hours remained at a higher level than the baseline in the control group, whereas that of the reduced expansion group returned to near baseline (Fig 4). The total plasma platinum concentrations at 72 hours were significantly higher in the control group compared with the reduced expansion group (P = .046). （R1, Q2）

The AUC at 0-24 hours for total plasma platinum concentrations of the reduced expansion group was 7.73 ± 4.94 μg hr/mL, while that of the control group was 2.16 ± 1.16 μg hr/mL (P = .039).

The mean platinum concentrations in the tumor at 1, 24 and 72 hours were 10.76 ± 2.57, 4.85 ± 1.31, and 2.79 ± 1.99 μg/g, respectively, in the reduced expansion group, and 1.57 ± 0.14, 3.76 ± 0.67, and 0.73 ± 0.11 μg/g, respectively, in the control group. The tumor platinum concentrations of the reduced expansion group at 1 hour were significantly higher than those of the control group (P = .044). The concentrations of the tumor platinum peaked at 1 hour in the reduced expansion group, while elevated at 24

hours in the control group (Fig 5).

There were no significant differences in any parameter of liver enzymes investigated between the two groups. In both groups, AST and ALT levels were elevated at 24 hours and decreased at 72 hours, and ALP level remained unchanged before and after TACE (Fig 6).

(R1, Q6, Q7) The mean tumor necrosis rates at 1, 24, and 72 hours were 14.4 ± 1.0, 86.1 ± 12.6, and 88.3 ± 14.9 %, respectively, in the reduced expansion group, while 19.7 ± 7.1, 74.2 ± 10.3, and 69.5 ± 9.3 %, respectively, in the control group. There were no significant differences between the two groups (P = .31, .25, and .21).

Discussion

Our in vitro study showed that 50 -100 μm dry SAP microspheres expanded to around 200 μm in size when in contact with a contrast material containing NaCl 2 w/v%. Although NaCl 2 w/v% suppressed the expansion more compared with 1 w/v%, NaCl 5 w/v% produced similar size SAP microspheres to 2 w/v%. These results show that there is a threshold for reduction of the level of expansion by using sodium ions containing water. The vendor information shows "when in contact with non-ionic contrast media or normal saline (NaCl 0.9 w/v%) before delivery, SAP microspheres expand to approximately 4x their dry state diameter" [13]. In our study, 50 -100 μm dry SAP microspheres were expanded to around 520 μm in size when in contact with non-ionic contrast material while around 230 μm in size when in contact with a contrast material containing NaCl 1 w/v%.

The expansion of cisplatin-loaded SAP microspheres was reduced to a similar level as that of unloaded SAP microspheres by using the reduced expansion technique. Based

on this result, the mechanism of the reduced expansion might not be influenced by the addition of cisplatin.(R2, Q14)

The tumor platinum concentrations in the reduced expansion group at 1 hour were approximately seven times higher than that of the control group. The increased volume of microspheres in the reduced expansion group was approximately one-tenth of the control expansion group (R2, Q1, Q3, Q15). Although the loaded volume of cisplatin per each particle decreased in the reduced expansion technique, a higher tumor tissue concentration of platinum could be achieved. The reasons could be considered that small size cisplatin-loaded microspheres penetrated into the tumor with fine feeders in the reduced expansion group much more than the control group. Although no statistically significant differences in the tumor necrosis rates were shown due to a limited number of animals. In general, a high platinum concentration in the tumor could achieve a higher tumor response rate although the values of platinum concentration in the tumor in this study could include the drug in the tissue and the microspheres. (R2, Q2, Q11)

The mixtures of cisplatin powder solution with SAP microspheres contained both cisplatin loaded SAP microspheres and cisplatin solution which were unloaded into the microspheres. Therefore, the plasma concentrations were related to not only release speeds, but also unloaded cisplatin doses, although there is no data of the comparison of release speeds between the reduced expansion and the control expansion groups (R1,Q3). The possibility of toxicities of the reduced expansion technique should be addressed due to the higher plasma concentration of platinum compared with the control technique. Previous clinical studies have shown the safety of arterial infusion of cisplatin solution without any drug delivery systems including drug-eluting microspheres [14, 15].(R1, Q4) Therefore, sever adverse events would rarely occur in patients even if the reduced

expansion technique is used.

SAP microspheres in the reduced expansion group were observed in the liver tumors and peripheral liver parenchyma in microscopic images. These results reflect that small size microspheres can contribute to the better distribution of microspheres and a higher drug concentration in non-hypervascular tumors such as liver metastases and cholangiocarcinoma in a clinical setting. The VX2 tumor has fine feeding arteries and could be suitable for a non-hypervascular tumors model [16-18].

In the control group, the plasma platinum C-max and the AUC at 0-24 hours were significantly lower, and the plasma concentration at 72 hours was higher than the reduced expansion group. In addition, the platinum tumor concentration at 24 hours in the control group was higher than the value at 1 hour. This was plausible because SAP microspheres by using the control expansion technique can load more cisplatin per one particle, therefore cisplatin-loaded SAP microspheres in the control group have a better ability to slowly release cisplatin. Further experimental study is needed to evaluate the cisplatin eluting speed in the reduced expansion and the control expansion groups. (R2, Q18)

There are some limitations in this study. First, SAP microspheres with a dry size of 50-100 μm were used. Further investigation using 30-60 μm dry-state SAP microspheres, which are currently the smallest available products, is needed to clarify the advantages of tiny microspheres. Second, we did not examine the drug release speeds in the in vitro study (R1, Q1; R2, Q2, Q12). Third, this study did not include bland-TAE using unloaded SAP microspheres group as a control arm. Fourth, this study included a limited number of animals. As a result, we were unable to prove statistically significant differences in the tumor necrosis rates among two groups. Fifth, the rabbits were observed for only 72 hours.

In conclusion, the expanding level of SAP microspheres was mostly reduced when in contact with fluid composed of a mixture of nonionic contrast material and 10 % NaCl at a 4:1 ratio. The reduced expanded cisplatin-loaded SAP microspheres achieved higher platinum concentrations in tumors although plasma concentration also increased (R1, Q8). In a clinical setting, cisplatin-loaded 50-100 μm SAP microspheres using the reduced expansion technique could be effective in TACE especially for non-hypervascular tumors.

Conflict of Interest Statement

Nippon Kayaku Co., Ltd. supported this study.Author 1 received a research grant to conduct this study from Nippon Kayaku Co.

Author 2 and Author 3 are employees of Nippon Kayaku Co., Ltd.
Author 4 is a trainer of Merit Medical and Nippon Kayaku Co., Ltd.
The other authors have no conflicts of interest and financial disclosures to declare.

Ethical Approval Statement

All applicable institutional and national guidelines for the care and use of animals were followed.

Informed Consent Statement

Does not apply.

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Figure 1.

Cumulative size distributions of SAP microspheres. The mean diameters of SAP microspheres expanded in Fluid A (NaCl 5 w/v%), Fluid B (NaCl 2 w/v%), Fluid C (NaCl 1 w/v%), and Fluid D (NaCl 0 w/v%) were 211.0, 198.3, 230.6, and 523.1 μm, respectively.

The swelling of SAP microspheres was mostly suppressed in Fluid B, and slightly increased in Fluid A and Fluid C.

Figure 2.

Cumulative size distributions of Cisplatin-loaded SAP microspheres. The mean diameters of cisplatin-loaded SAP microspheres were 188.4 μm in the reduced expansion technique

and 404.9 μm in the control expansion technique, with significant difference ($P < .01$).

Figure 3.

Microscopic images (× 100 magnification) of cisplatin-loaded SAP microspheres. The reduced expansion technique (a) produced about half the size of cisplatin-loaded SAP microspheres compared with the control expansion technique (b).

Figure 4.

Concentrations of total platinum in plasma after administration of cisplatin. Total plasma platinum concentrations remained higher within the first 24 hours in the reduced expansion group than the control group, while remained at a higher level than the baseline at 72 hours in the control group.

Figure 5.

Concentrations of platinum in the tumor tissue after administration of cisplatin. The mean platinum concentrations in VX2 tumor at 1, 24 and 72 hours were 10.76 ± 2.57, 4.85 ± 1.31, and 2.79 ± 1.99 μg/g, respectively, in the reduced expansion group, and 1.57 ± 0.14, 3.76 ± 0.67, and 0.73 ± 0.11 μg/g, respectively, in the control group. The tumor platinum concentrations of the reduced expansion group at 1 hour was significantly higher than those of the control group (*$P = .044$).

Figure 6.

Changes in liver enzymes of plasma after transarterial chemoembolization. There was no significant difference in any parameter investigated between the two groups.

Table 1 Mean diameter of microspheres related to component of the fluids

	Dry	Fluid A	Fluid B	Fluid C	Fluid D	Reduced Expansion	Control Expansion
Iohexol (% V/V)	-	50	80	90	100	80	80
NaCl (% w/v)	-	5	2	1	0	2	0.18
Cisplatin (mg/mL)	-	-	-	-	-	4	4
Mean Diameter (µm)	79.9	211.0	198.3	230.6	521.3	188.4	404.9

Figure 1

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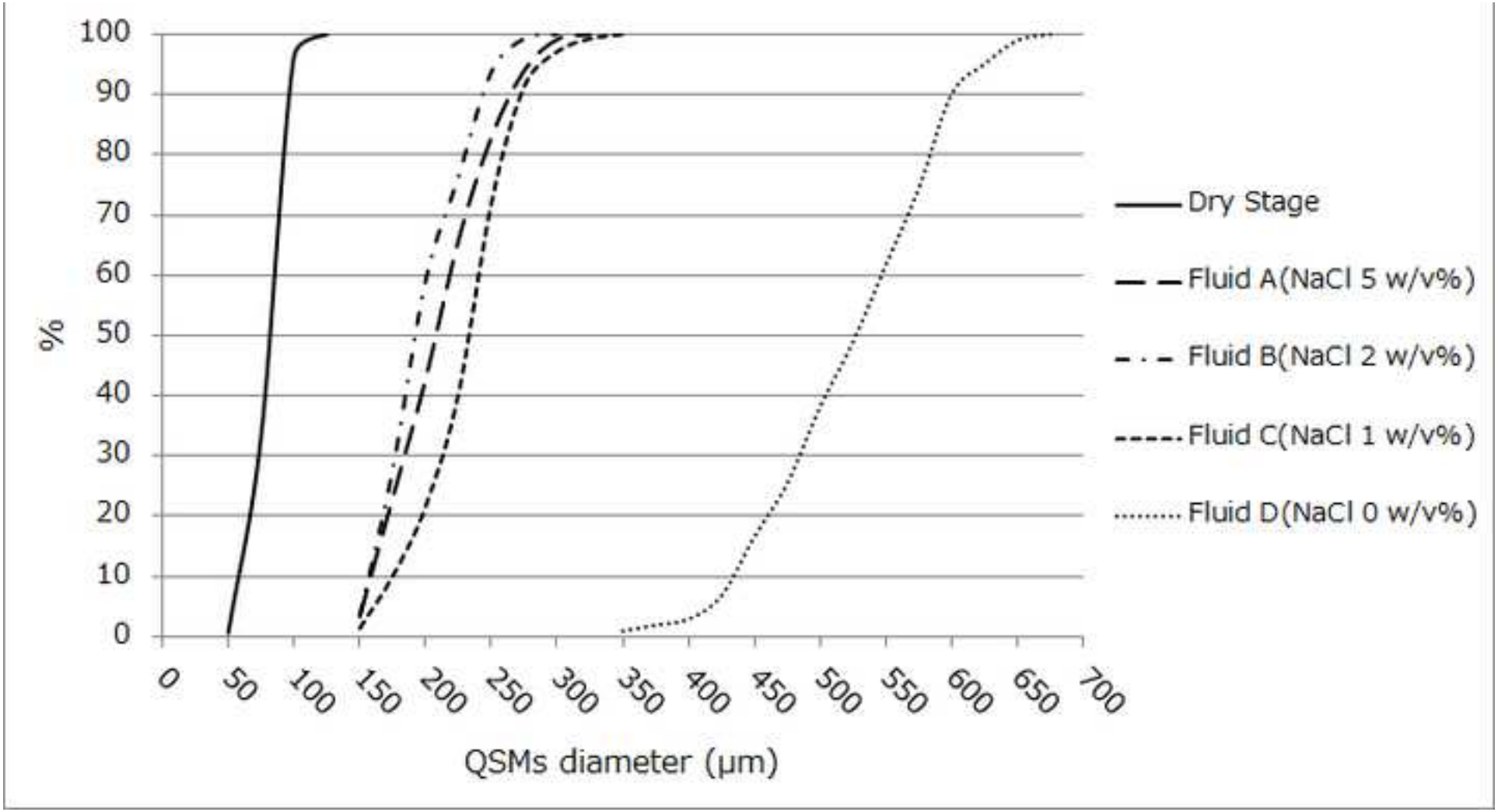

Figure 2

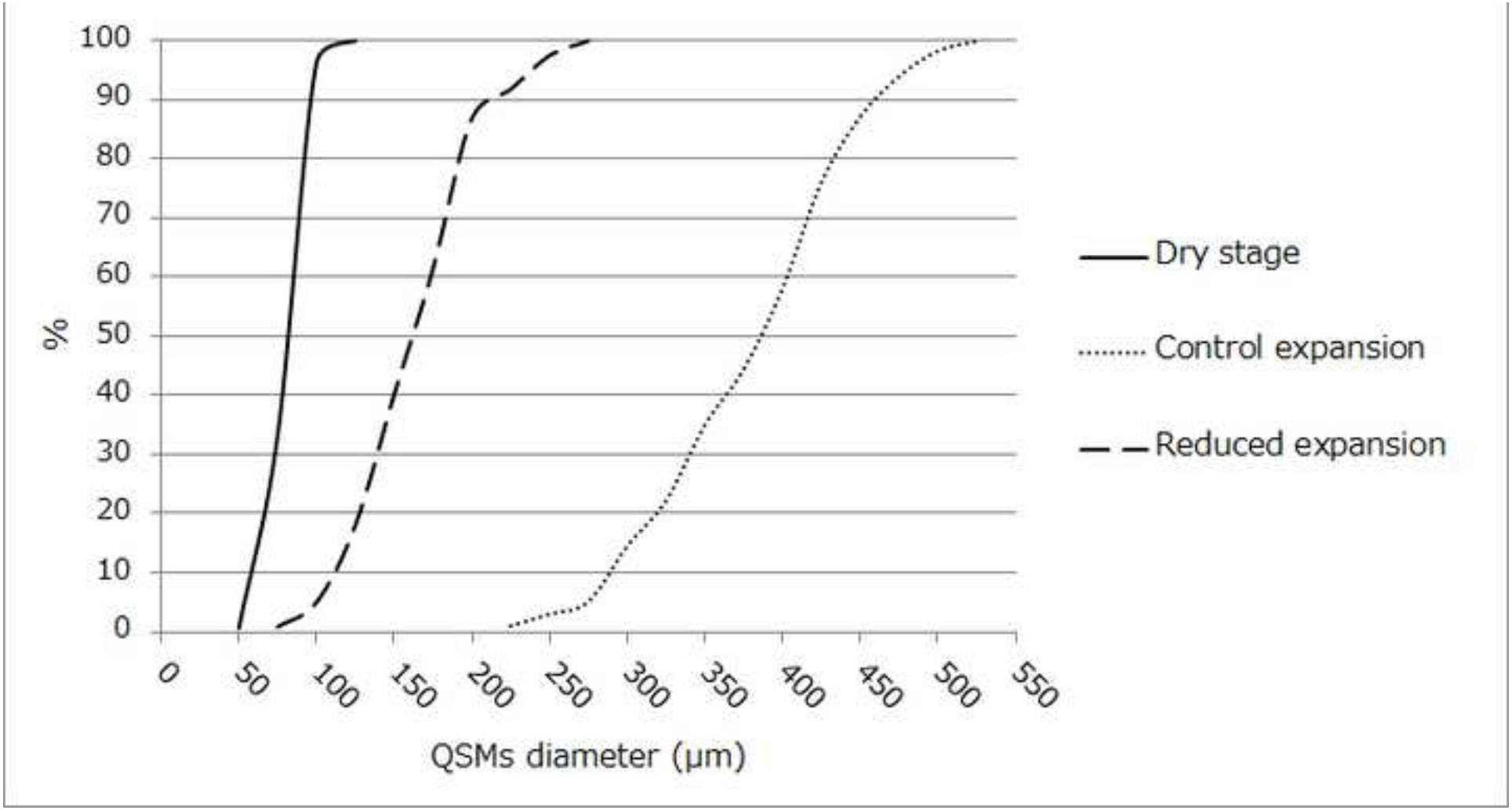

Figure 3a

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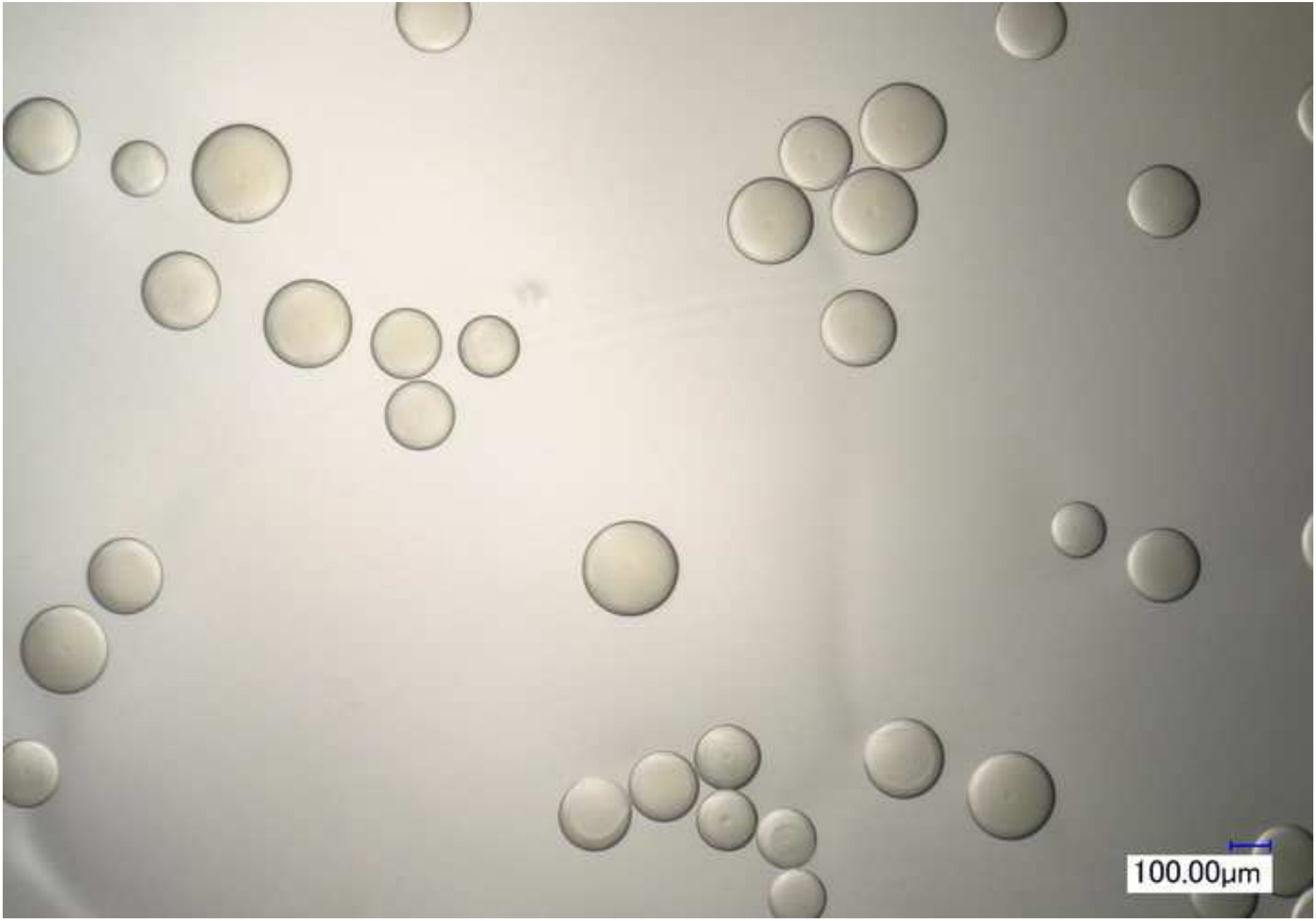

Figure 3b

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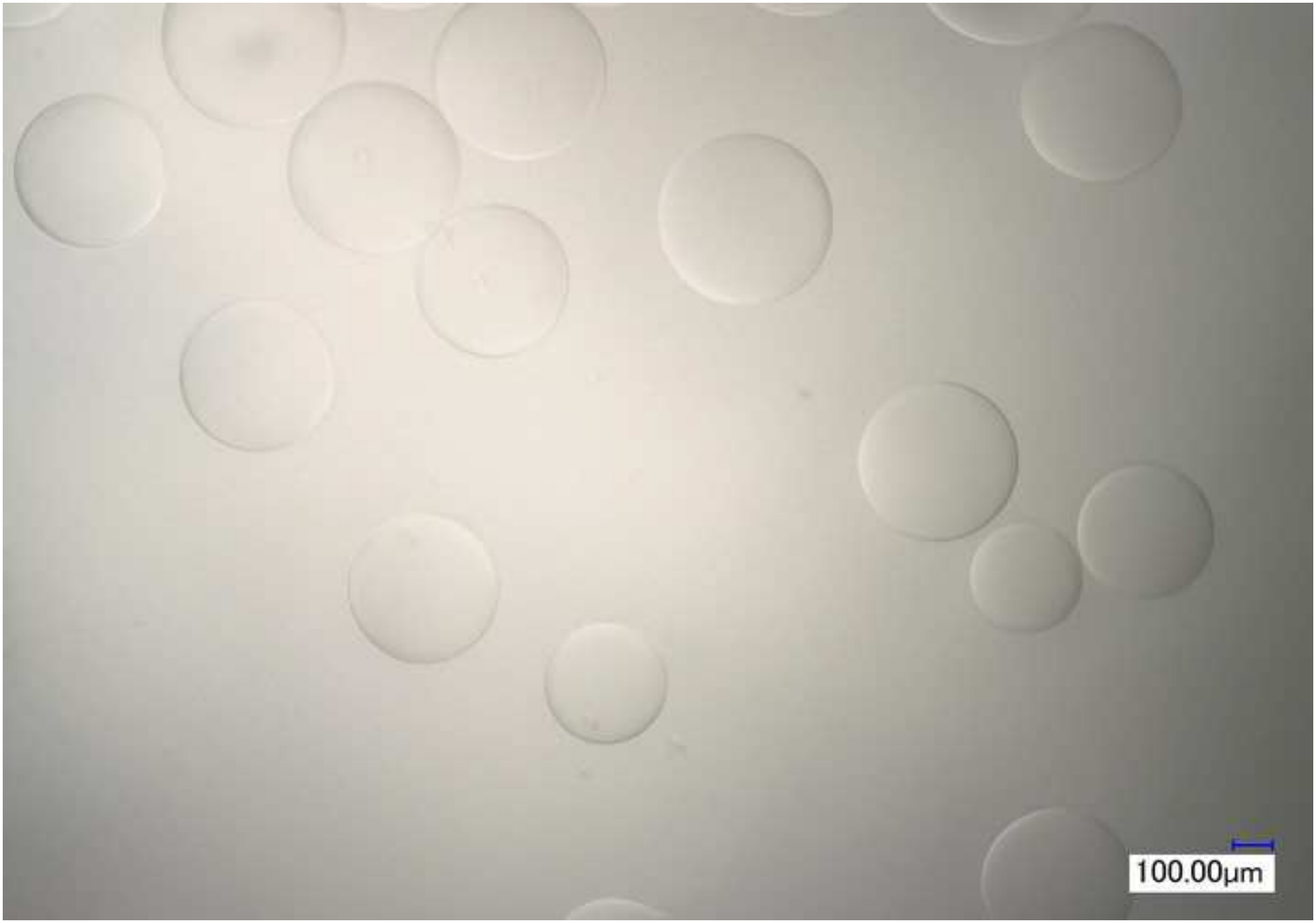

Figure 4

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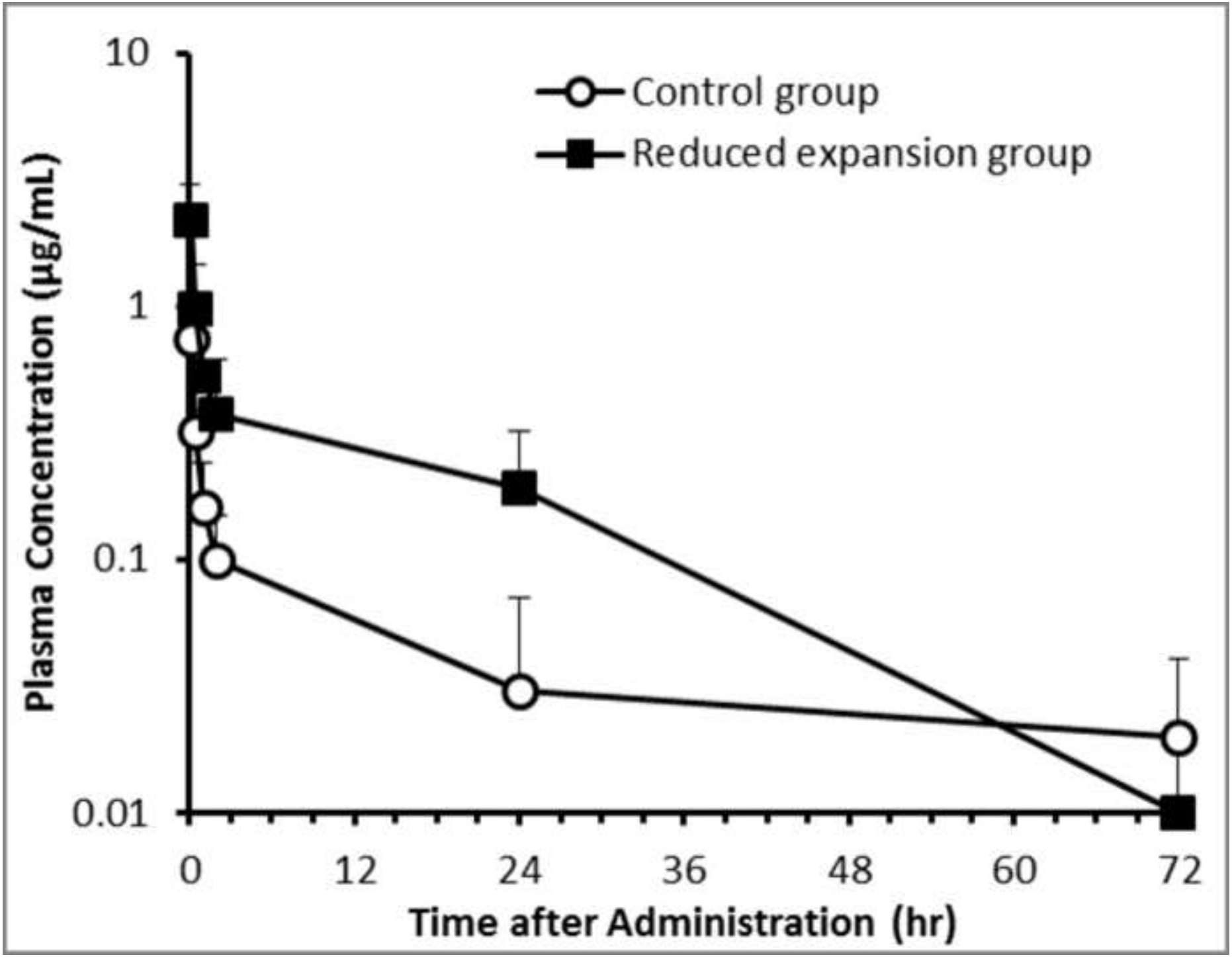

Figure 5

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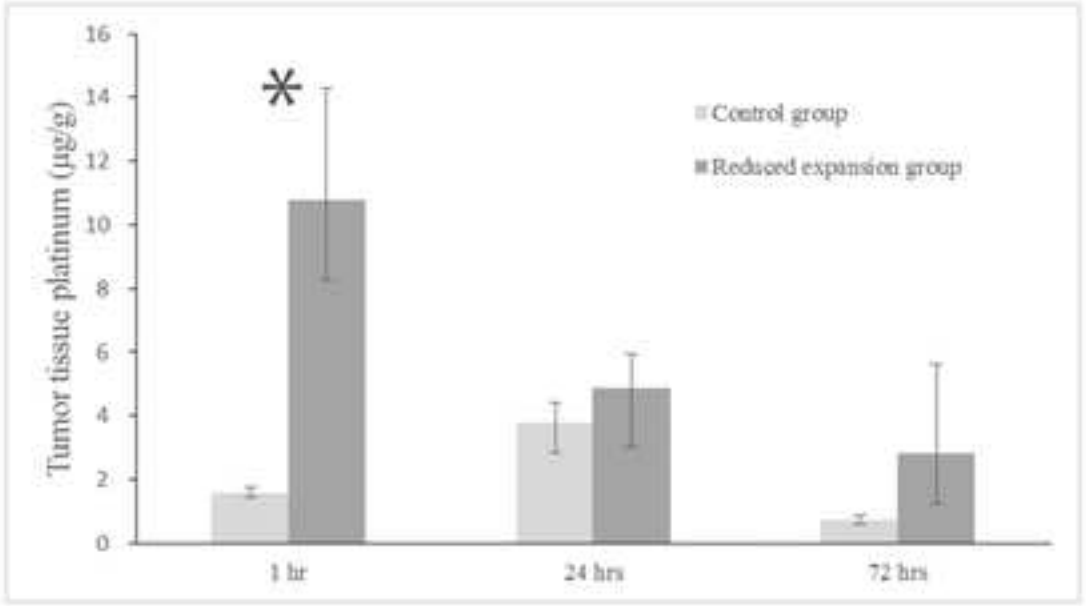

Figure 6

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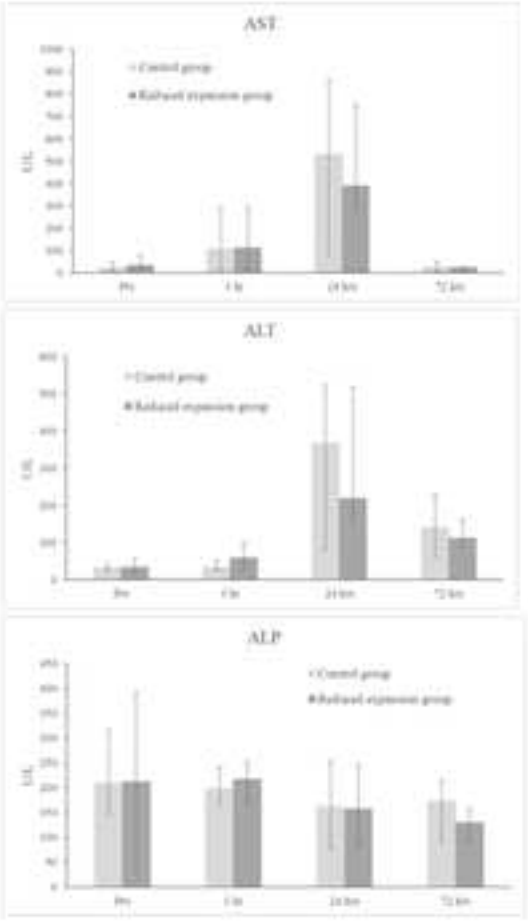

Abstract

Purpose:

To analyze size changes of super absorbent polymer (SAP) microspheres with the reduced expansion technique, and to evaluate pharmacological advantages of transarterial chemoembolization using cisplatin-loaded SAP microspheres with the reduced expansion technique.

Materials and Methods:

In an in vitro study, diluted contrast materials containing different concentrations of sodium ions were examined to expand SAP microspheres and determined the reduced expansion technique. Size distributions of cisplatin-loaded SAP microspheres were analyzed. In an in vivo study, TACE was performed using cisplatin-loaded SAP microspheres with the reduced expansion and control techniques in 18 VX2 rabbits.

Results:

The degree of expansion was reduced to the greatest extent by using a mixture of nonionic contrast material and 10 % NaCl at a 4:1 ratio (NaCl 2 w/v%). The mean diameter of the reduced expansion of cisplatin-loaded SAP microspheres was 188.4 μm, while that of the control expansion was 404.9 μm. The plasma concentrations of the reduced expansion group at 5 minutes after TACE were significantly higher than those of the control expansion group (2.19 ± 0.77 *vs* 0.75 ± 0.08 μg/mL, P = .01). The tumor platinum concentrations of the reduced expansion group at 1 hour were significantly higher than those of the control expansion group (10.76 ± 2.57 *vs* 1.57 ± 0.14 μg/g, P = .044).

Conclusion:

The expanding level of SAP microspheres can be reduced by using hypertonic saline. Cisplatin-loaded SAP microspheres with the reduced expansion technique have the advantages of achieving higher cisplatin tissue concentration in TACE for liver tumors.

Introduction

Drug-eluting microspheres have been developed and often used in transarterial chemoembolization (TACE) for liver tumors [1-5]. Super absorbent polymer (SAP) microspheres (HepaSphere; Merit Medical, South Jordan, Utah, USA) are the only microspheres that can load cisplatin by the unique characteristic of mechanical absorption without an ion-exchange process [6].

SAP microspheres are a hydrophilic copolymer with the property of expansion by the absorption of water in the microspheres [7, 8]. Regarding the mechanism of the absorption, when SAP microspheres contact with water, sodium ions ionize away from the carboxyl groups into the microspheres and the ion concentration difference occurs between the inside and the outside of the hydrophilic copolymer of SAP microspheres. Consequently, the water is absorbed into the hydrophilic copolymer and the microspheres expand.

It is known that SAP microspheres mixed with nonionic contrast medium expand approximately four times larger than original sizes in the dry stage [9]. Theoretically, in sodium ions containing water, the hydrophilic copolymer could absorb less water due to little ion concentration difference between the inside and the outside of the hydrophilic copolymer. As a result, the expansion level could be reduced compared with the non-sodium ionic water.

The smaller size microspheres should be ideal for tumors with fine feeding arteries allowing deep penetration into the tumor [10-12]. However, it remains a dilemma whether the amount of cisplatin-loaded in microspheres could be reduced in the less expanded microspheres.

Based on the above background, firstly an in vitro study was conducted to compare the level of expansion of SAP microspheres in various concentrations of sodium ions containing solvents, and the size distribution of cisplatin-loaded SAP microspheres produced by the reduced expansion technique was examined. Secondly, an in vivo study of TACE using a rabbit VX2 liver tumor model was conducted to compare the pharmacokinetics findings of cisplatin-loaded SAP microspheres between the reduced and control expansion techniques.

Materials and Methods

In Vitro Study of Expanding Levels of SAP Microspheres

In the first part of the in vitro study, the following four types of diluted or non-diluted contrast materials containing different concentrations of sodium ions (Fluid A, B, C, and D) were prepared at room temperature to compare the level of expansion of SAP microspheres. The total volume of each fluid was 10 mL. Fluid A was composed of 5mL of Iohexol 350 mg I/mL (Omunipaque; Daiichi Sankyo, Tokyo, Japan) and 5 mL of 10 % NaCl (NaCl 5 w/v%), Fluid B was 8 mL of Iohexol and 2 mL of 10 % NaCl (NaCl 2 w/v%), Fluid C was 9 mL of Iohexol and 1 mL of 10 % NaCl (NaCl 1 w/v%), and Fluid D was 10 mL of Iohexol (NaCl 0 w/v%). SAP microspheres with a dry size of 50-100 μm were expanded in each fluid for 15 minutes. Around 100 expanded microspheres were randomly sampled and the mean diameters and the size distribution were evaluated using a digital microscope (VHX-1000; Keyence, Osaka, Japan). Then, the fluid which mostly suppressed swelling of SAP microspheres was defined as the reduced expansion techniques.

In the second part of the in vitro study, cisplatin-loaded SAP microspheres created using the above-reduced expansion techniques were evaluated. A fine-powder formulation of cisplatin at a dose of 50 mg (IA-Call; Nippon Kayaku, Tokyo, Japan) was dissolved in 10 mL of Iohexol 350 mg I/mL at about 40℃. The cisplatin solution was mixed with 10% NaCl using the ratio of the reduced expansion technique according to the result of the first part of the in vitro study. Then, SAP microspheres with a dry size of 50-100 μm were expanded using this mixture, which was defined as the reduced expansion of cisplatin-loaded SAP microspheres. In addition, the cisplatin solution was mixed with physiological saline (0.9% NaCl) instead of 10 % NaCl using the same ratio and SAP microspheres were expanded, which was defined as the control expansion of cisplatin-loaded SAP microspheres. The size distributions of both cisplatin-loaded SAP microspheres were analyzed and microscopic examinations were conducted.

In Vivo Animal Study

The study protocol was approved by the Animal Experimentation Committee of our institution, and all experiments were performed in accordance with the Animal Care Guidelines of our institution. New Zealand white rabbits weighing 2.90–4.15 kg (mean 3.44 kg) were purchased from Japan SLC Inc. (Hamamatsu, Japan). VX2 tumors were implanted in the left lobe of the livers under laparotomy two weeks prior to TACE.

Eighteen rabbits with VX2 liver tumors were divided into two groups: the reduced expansion (n=9) group and the control (n=9) group. The reduced expansion and the control expansion of cisplatin-loaded SAP microspheres were prepared according to the results of the second part of the in vitro study.

Cisplatin-loaded SAP microspheres were injected via a 1.7 Fr microcatheter into

the left hepatic artery under fluoroscopic guidance in an angiography suite (Surginix; Toshiba, Otahara, Japan). The endpoint of the injection in both groups was a stasis of the left hepatic arterial flow.

The plasma concentrations of platinum were measured with an atomic absorption spectrometry (AAS) before the treatment and 5 minutes, 0.5, 1, 2, 24, and 72 hours after TACE. Plasma ultrafiltrate or diluted plasma was directly introduced to the AAS instrument. For tissues, samples were wet-ashed by nitric acid and the platinum was extracted as diethyldithiocarbamate-platinum complex using a chloroform. The extracted platinum complex was applied to the AAS. In the plasma ultrafiltrate, limit of detection (LOD) and lower limit of quantification (LOQ) were 0.002 and 0.01 μg/mL, respectively. In the non-filtered plasma, LOD and LOQ were 0.01 and 0.05 μg/mL, respectively. In the tissues, LOD and LOQ were 0.017 and 0.033 μg/g tissue, respectively.

The serum level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured before the treatment and 1, 24 and 72 hours after TACE. All rabbits were euthanized with an overdose of pentobarbital at 1 hour (n=3), 24 hours (n=3), and 72 hours (n=3) after TACE in both groups.

The liver tumors were cut in half along the midline and half were immediately frozen to measure the tumor platinum concentration. The remaining half was embedded in paraffin and stained with hematoxylin and eosin for histopathological evaluation. Tumor necrosis rates at 1, 24 and 72 hours were calculated as a percentage of the tumor necrosis area for each slice by an independent pathologist blinded to the treatments. The tumor necrosis ratio was estimated by visual calculation.

Statistical Analysis

All in vivo study data were provided as arithmetic mean ± SD. Pairwise comparisons of these values between the reduced expansion and the control groups were performed with Student's t-test. Values of $p < .05$ were considered significant. These analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, Ill. USA). The area under the concentration-time curve (AUC) calculations of the plasma concentration of platinum were performed using Phoenix WinNonlin (Certara G.K.; Princeton, NJ, USA).

Results

In Vitro Diameter Change of SAP Microspheres

Mean (min - max) diameter of SAP microspheres in dry-state was 79.9 (47.7 – 106.1) μm, and in the Fluids A, B, C and D were 211.0 (133.8 – 313.2) μm, 198.3 (130.2 – 280.4) μm, 230.6 (135.3 – 344.8) μm, 523.1 (342.7 – 653.5) μm, respectively. The cumulative size distributions are shown in Fig 1. The swelling of SAP microspheres was mostly suppressed in Fluid B. Therefore, the mixture using 8 mL of non-ionic contrast material and 2 mL of 10 % NaCl (NaCl 2 w/v%) was determined as the reduced expansion technique.

According to the above results, the reduced expansion of cisplatin-loaded SAP microspheres was produced using a cisplatin powder solution dissolved by 8 mL of Iohexol combined with 2 mL of 10% NaCl. The control expansion was produced using a cisplatin powder solution dissolved by 8 mL of Iohexol combined with 2 mL of saline. The mean (min - max) diameter of the reduced expansion of cisplatin-loaded SAP microspheres was 188.4 (82.2 – 298.5) μm, while that of the control expansion was 404.9 (220.6 – 566.9) μm (Table 1). The mean size of the reduced expansion of cisplatin-loaded SAP microspheres was 2.4 times larger than that of the dry size of SAP microspheres,

while that of the control expansion was 5.1 times larger than the dry size. The cumulative size distributions are shown in Fig 2. The microscopic findings showed calibrated and spherical shapes of cisplatin-loaded SAP microspheres, which were consistent with the results of the measurement of the sizes (Fig 3).

In Vivo Pharmacological and Histological Findings

The mean administered doses of cisplatin were 1.01 ± 0.19 mg/kg in the reduced expansion group and 0.90 ± 0.17 mg/kg in the control group (P = .26).

The total platinum concentrations in plasma peaked after 5 minutes in both groups. The maximum concentrations (C-max) were 2.19 ± 0.77 μg/mL in the reduced expansion group and 0.75 ± 0.08 μg/mL in the control group (P = .01). The total plasma platinum concentrations at 72 hours remained at a higher level than the baseline in the control group, whereas that of the reduced expansion group returned to near baseline (Fig 4). The total plasma platinum concentrations at 72 hours were significantly higher in the control group compared with the reduced expansion group (P = .046).

The AUC at 0-24 hours for total plasma platinum concentrations of the reduced expansion group was 7.73 ± 4.94 μg hr/mL, while that of the control group was 2.16 ± 1.16 μg hr/mL (P = .039).

The mean platinum concentrations in the tumor at 1, 24 and 72 hours were 10.76 ± 2.57, 4.85 ± 1.31, and 2.79 ± 1.99 μg/g, respectively, in the reduced expansion group, and 1.57 ± 0.14, 3.76 ± 0.67, and 0.73 ± 0.11 μg/g, respectively, in the control group. The tumor platinum concentrations of the reduced expansion group at 1 hour were significantly higher than those of the control group (P = .044). The concentrations of the tumor platinum peaked at 1 hour in the reduced expansion group, while elevated at 24

hours in the control group (Fig 5).

There were no significant differences in any parameter of liver enzymes investigated between the two groups. In both groups, AST and ALT levels were elevated at 24 hours and decreased at 72 hours, and ALP level remained unchanged before and after TACE (Fig 6).

The mean tumor necrosis rates at 1, 24, and 72 hours were 14.4 ± 1.0, 86.1 ± 12.6, and 88.3 ± 14.9 %, respectively, in the reduced expansion group, while 19.7 ± 7.1, 74.2 ± 10.3, and 69.5 ± 9.3 %, respectively, in the control group. There were no significant differences between the two groups (P = .31, .25, and .21).

Discussion

Our in vitro study showed that 50 -100 μm dry SAP microspheres expanded to around 200 μm in size when in contact with a contrast material containing NaCl 2 w/v%. Although NaCl 2 w/v% suppressed the expansion more compared with 1 w/v%, NaCl 5 w/v% produced similar size SAP microspheres to 2 w/v%. These results show that there is a threshold for reduction of the level of expansion by using sodium ions containing water. The vendor information shows “when in contact with non-ionic contrast media or normal saline (NaCl 0.9 w/v%) before delivery, SAP microspheres expand to approximately 4x their dry state diameter” [13]. In our study, 50 -100 μm dry SAP microspheres were expanded to around 520 μm in size when in contact with non-ionic contrast material while around 230 μm in size when in contact with a contrast material containing NaCl 1 w/v%.

The expansion of cisplatin-loaded SAP microspheres was reduced to a similar level as that of unloaded SAP microspheres by using the reduced expansion technique. Based

on this result, the mechanism of the reduced expansion might not be influenced by the addition of cisplatin.

The tumor platinum concentrations in the reduced expansion group at 1 hour were approximately seven times higher than that of the control group. The increased volume of microspheres in the reduced expansion group was approximately one-tenth of the control expansion group. Although the loaded volume of cisplatin per each particle decreased in the reduced expansion technique, a higher tumor tissue concentration of platinum could be achieved. The reasons could be considered that small size cisplatin-loaded microspheres penetrated into the tumor with fine feeders in the reduced expansion group much more than the control group. Although no statistically significant differences in the tumor necrosis rates were shown due to a limited number of animals. In general, a high platinum concentration in the tumor could achieve a higher tumor response rate although the values of platinum concentration in the tumor in this study could include the drug in the tissue and the microspheres.

The mixtures of cisplatin powder solution with SAP microspheres contained both cisplatin loaded SAP microspheres and cisplatin solution which were unloaded into the microspheres. Therefore, the plasma concentrations were related to not only release speeds, but also unloaded cisplatin doses, although there is no data of the comparison of release speeds between the reduced expansion and the control expansion groups. The possibility of toxicities of the reduced expansion technique should be addressed due to the higher plasma concentration of platinum compared with the control technique. Previous clinical studies have shown the safety of arterial infusion of cisplatin solution without any drug delivery systems including drug-eluting microspheres [14, 15]. Therefore, sever adverse events would rarely occur in patients even if the reduced

expansion technique is used.

SAP microspheres in the reduced expansion group were observed in the liver tumors and peripheral liver parenchyma in microscopic images. These results reflect that small size microspheres can contribute to the better distribution of microspheres and a higher drug concentration in non-hypervascular tumors such as liver metastases and cholangiocarcinoma in a clinical setting. The VX2 tumor has fine feeding arteries and could be suitable for a non-hypervascular tumors model [16-18].

In the control group, the plasma platinum C-max and the AUC at 0-24 hours were significantly lower, and the plasma concentration at 72 hours was higher than the reduced expansion group. In addition, the platinum tumor concentration at 24 hours in the control group was higher than the value at 1 hour. This was plausible because SAP microspheres by using the control expansion technique can load more cisplatin per one particle, therefore cisplatin-loaded SAP microspheres in the control group have a better ability to slowly release cisplatin. Further experimental study is needed to evaluate the cisplatin eluting speed in the reduced expansion and the control expansion groups.

There are some limitations in this study. First, SAP microspheres with a dry size of 50-100 μm were used. Further investigation using 30-60 μm dry-state SAP microspheres, which are currently the smallest available products, is needed to clarify the advantages of tiny microspheres. Second, we did not examine the drug release speeds in the in vitro study. Third, this study did not include bland-TAE using unloaded SAP microspheres group as a control arm. Fourth, this study included a limited number of animals. As a result, we were unable to prove statistically significant differences in the tumor necrosis rates among two groups. Fifth, the rabbits were observed for only 72 hours.

In conclusion, the expanding level of SAP microspheres was mostly reduced when in contact with fluid composed of a mixture of nonionic contrast material and 10 % NaCl at a 4:1 ratio. The reduced expanded cisplatin-loaded SAP microspheres achieved higher platinum concentrations in tumors although plasma concentration also increased. In a clinical setting, cisplatin-loaded 50-100 μm SAP microspheres using the reduced expansion technique could be effective in TACE especially for non-hypervascular tumors.

Conflict of Interest Statement

Nippon Kayaku Co., Ltd. supported this study. Author 1 received a research grant to conduct this study from Nippon Kayaku Co.
Author 2 and Author 3 are employees of Nippon Kayaku Co., Ltd.
Author 4 is a trainer of Merit Medical and Nippon Kayaku Co., Ltd.
The other authors have no conflicts of interest and financial disclosures to declare.

Ethical Approval Statement

All applicable institutional and national guidelines for the care and use of animals were followed.

Informed Consent Statement

Does not apply.

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Figure 1.

Cumulative size distributions of SAP microspheres. The mean diameters of SAP microspheres expanded in Fluid A (NaCl 5 w/v%), Fluid B (NaCl 2 w/v%), Fluid C (NaCl 1 w/v%), and Fluid D (NaCl 0 w/v%) were 211.0, 198.3, 230.6, and 523.1 μm, respectively.

The swelling of SAP microspheres was mostly suppressed in Fluid B, and slightly increased in Fluid A and Fluid C.

Figure 2.

Cumulative size distributions of Cisplatin-loaded SAP microspheres. The mean diameters of cisplatin-loaded SAP microspheres were 188.4 μm in the reduced expansion technique

and 404.9 μm in the control expansion technique, with significant difference ($P < .01$).

Figure 3.

Microscopic images (× 100 magnification) of cisplatin-loaded SAP microspheres. The reduced expansion technique (a) produced about half the size of cisplatin-loaded SAP microspheres compared with the control expansion technique (b).

Figure 4.

Concentrations of total platinum in plasma after administration of cisplatin. Total plasma platinum concentrations remained higher within the first 24 hours in the reduced expansion group than the control group, while remained at a higher level than the baseline at 72 hours in the control group.

Figure 5.

Concentrations of platinum in the tumor tissue after administration of cisplatin. The mean platinum concentrations in VX2 tumor at 1, 24 and 72 hours were 10.76 ± 2.57, 4.85 ± 1.31, and 2.79 ± 1.99 μg/g, respectively, in the reduced expansion group, and 1.57 ± 0.14, 3.76 ± 0.67, and 0.73 ± 0.11 μg/g, respectively, in the control group. The tumor platinum concentrations of the reduced expansion group at 1 hour was significantly higher than those of the control group (*$P = .044$).

Figure 6.

Changes in liver enzymes of plasma after transarterial chemoembolization. There was no significant difference in any parameter investigated between the two groups.