Design and evaluation of a novel flavonoid-based radioprotective agent utilizing monoglucosyl rutin

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ABSTRACT

In this study, three novel flavonoid composite materials, created by combining an aglycone [quercetin (QUE), hesperetin (HES) or naringenin (NAR)] with monoglucosyl rutin (MGR), were designed to test for improved radioprotectivity compared with that provided by administration of MGR alone. Aglycone in the MGR-composite state was highly soluble in water, compared with aglycone alone dissolved in dimethyl sulfoxide or distilled water. The antioxidant activity of the three flavonoid composites was as high as that of MGR only. Next, the cytotoxicity test after 30 min treatment of an MGR composite showed a clear reduction in cell viability and suggested that a rapid introduction of aglycone into cells had taken place. In addition, QUE/MGR and HES/MGR composites strongly scavenged intracellular reactive oxygen species (ROS) induced by X-ray irradiation as well as MGR alone did. However, in the colony-formation assay using irradiated Chinese hamster ovary (CHO) cells, the HES/MGR composite showed a stronger radioprotective effect than MGR alone did, but the QUE/MGR composite showed no additional protective effect compared with the control. Furthermore, it was revealed that QUE and QUE/MGR composite treatment had the effect of reducing the glutathione (GSH) content in cells, and that QUE showed a stronger inhibition of PARP activity compared that of HES and NAR. Our data demonstrated that when designing a flavonoid composite as a radioprotective agent, it was necessary to select an appropriate aglycone, considering not only its antioxidant ability but also its inhibitory effect on cell recovery or DNA repair after radiation injury.

Keywords: flavonoid; aglycone; monoglucosyl-rutin; flavonoid composite; solubilizing effect; irradiation damage; radioprotective agent

INTRODUCTION

Rutin is a natural flavonoid contained in fruits and vegetables and is one of the glycosulated derivatives of quercetin (QUE) [1]. Quercetin and rutin, and many other flavonoids, have a high antioxidant activity and thus have been widely studied in the fields of food science and nutrition science [2]. However, the extremely low water solubility of these flavonoids (quercetin: <10 mg/l, rutin: <130 mg/l at room temperature) is regarded as an obstacle in various studies and developments [3–6]. In order to overcome this problem of low water solubility, monoglucosyl rutin (MGR), wherein glucose is added to rutin using glycosyltransferase, has been proposed [7]. MGR has remarkably improved water solubility compared with the original rutin, but has lower biological availability than rutin because of inefficient incorporation into cells [8, 9]. In order to solve this problem, a method for improving biological availability has been recently proposed: preparing a mixture of MGR and a hardly water-soluble substance, utilizing the solubilizing effect of MGR. It was reported that a hardly water-soluble substance and MGR in aqueous solution...
formed micelle-like nanostructures of about several nanometers in diameter due to hydrophobic interaction. However, unlike ordinary surfactants, MGR has been reported to have a low surface activity effect and is therefore not a simple micelle structure, but a special nanostructure. This composite, which is a mixture of MGR and a hardly water-soluble substance, achieves not only the solubilization of the hardly water-soluble substance by MGR, but also protection and stabilization of the hardly water-soluble substance by the functionality of MGR (such as antioxidant potency and the ultraviolet absorption effect), and so is a composite having additional functionality compared with ordinary solubilizing agents and surfactants [10–12].

Further more, flavonoids are known to be naturally occurring radioprotectors. Moreover, patients of radiotherapy and radiation workers might benefit from consuming flavonoids daily to reduce their radiation damage risk [13]. For example, flavonoid mixtures containing rutin or quercetin reduced the frequency of micronucleated reticulocytes in the peripheral blood of irradiated mice [14]. Also, genistein, a flavonoid contained in leguminous plants such as soybean, has been reported in clinical trials to mitigate side effects during radiation therapy [15].

Considering the radioprotective effect of flavonoids with respect to low-dose irradiation, it is noted that flavonoids are radical scavengers and suppress the generation of intracellular reactive oxygen species (ROS) caused by radiation [16]. It is therefore desirable that flavonoids selected as radioprotective agents have high antioxidant activity; on the other hand, it is also necessary to consider the influence of the adverse pro-oxidant effect that oxidized flavonoids can have on cells [17]. As a new application of MGR, its use as a radioprotective agent has been investigated in recent years, and its radioprotective effect has been confirmed in a test using mammalian cells [18]. However, it has been revealed that, when used singly, MGR has to be at a high concentration in order to exhibit its radioprotective effect [18].

In this paper, based on the idea that the difficulty in using MGR as a radioprotective agent resides in the low efficiency of incorporation of MGR into cells, an attempt has been made to solve this problem by using a flavonoid composite [a combination of MGR and a hardly water-soluble flavonoid (aglycone)]. We thus aimed to develop a novel radioprotective agent using safe and functional MGR in combination with natural flavonoids.

MATERIALS AND METHODS

Reagents

MGR was provided by Toyo Sugar Refining Co., Ltd (Tokyo, Japan). Quercetin (QUE dihydrate; 338.27 g/mol), hesperetin (HES; 302.28 g/mol) and naringenin (NAR; 272.25 g/mol) were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Dimethyl sulfoxide (DMSO; 78.13 g/mol), 1,1-diphenyl-2-picrylhydrazyl (DPPH; 394.32 g/mol) free radical and L-ascorbic acid (AA; 176.12 g/mol) were purchased from the Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). The other reagents and solvents used were of reagent grade. Figure 1 shows the chemical structures of flavonoids and other reagents used in this study.
and molar mass of the flavonoids and L-ascorbic acid used in this study.

**Cell culture and irradiation**

CHO-K1 (Chinese hamster ovary-K1) cells were kindly provided by RIKEN RCB (Tsukuba, Japan) and cultured in a medium prepared by adding 10% fetal bovine serum (FBS) and 1% antibiotics and antimycotics (Gibco, Indianapolis, MO) to alpha minimum essential medium (αMEM) at 37°C in the presence of 5% CO2. All the experiments were carried out using cells in a logarithmic growth phase.

Irradiation was carried out using the X-ray generator TITAN 320 (200 kVp, 20 mA, 5 mm aluminum and copper filter, Shimadzu, Tokyo, Japan) at room temperature. The exposure rate was 1 Gy/min.

**Preparation of flavonoid composites utilizing spray-drying**

Each flavonoid composite was prepared by mixing a hardly water-soluble flavonoid (aglycone) and MGR at a molar ratio of 1:4, dissolving the mixture in a 8:2 mixed solution of ethanol:water, and pulverizing the resulting mixed solution using a spray dryer (CPL-2, Ohkawara Kakohki, Kanagawa, Japan). The inlet temperature of a drying chamber was 120°C, the outlet temperature was 68°C, and the spraying air pressure was 0.2 MPa. The spray-dried powder was stored in a desiccator for sufficient drying before use in a test [10, 11, 19]. The combinations of three kinds of flavonoid composite prepared in this experiment are shown in Table 1.

**Measurement of water solubility using high-performance liquid chromatography**

Aqueous solutions of MGR and the flavonoid composites (QUE/MGR, HES/MGR, NAR/MGR) mentioned above were prepared by dissolving with only distilled water (DW). On the other hand, aglycones were dissolved in DMSO at 50 mM and then diluted with distilled water to adjust to 1% DMSO for the final concentration. All aglycone solutions were adjusted to 100 μM each in molar concentration, and the flavonoid composite solutions were adjusted to an average molar concentration of 500 μM each, so that the aglycone contained therein was 100 μM in molar concentration. Next, each sample was stirred by a vortex mixer for 1 min or more, sampled in a 10 ml syringe equipped with a 0.2 μm filter at each measurement time (5, 10, 20, 40, 60 min), and followed up with pressure filtration. After adding acetonitrile at the same ratio as in the mobile phase to the sample passed through the filter, the peak area (μVs) at the maximum absorption wavelength of aglycone (QUE: 370 nm, HES: 285 nm, NAR: 290 nm) was measured using high-performance liquid chromatography (HPLC) apparatus (Alliance 2695, Waters, MA, USA). The aglycone concentration was adjusted to 100 μM each in molar concentration, and the flavonoid composite solutions were adjusted to an average molar concentration of 500 μM each, so that the aglycone contained therein was 100 μM in molar concentration.

**Table 1. Combination of three types of flavonoid composites prepared in this experiment**

| Flavonoid composite | Aglycone       | Glucosyl flavonoid |
|---------------------|----------------|--------------------|
| 1 QUE/MGR           | Quercetin(QUE) | Monoglucosyl rutin(MGR) |
| mixing ratio:QUE:MGR = 1:4(molar weight ratio) | average molar mass:681.42 g/mol |                |
| 2 HES/MGR           | Hesperetin(HES) | Monoglucosyl rutin(MGR) |
| mixing ratio:HES:MGR = 1:4(molar weight ratio) | average molar mass:681.43 g/mol |
| 3 NAR/MGR           | Naringenin(NAR) | Monoglucosyl rutin(MGR) |
| mixing ratio:NAR:MGR = 1:4(molar weight ratio); average molar mass:675.43 g/mol |

QUE = quercetin, MGR = monoglucosyl rutin, HES = hesperetin, NAR = naringenin.
Determined by obtaining a calibration curve from the peak areas (μVs) of the standard samples (QUE, HES and NAR; 500 μM each). The measurement conditions of the HPLC apparatus used were as follows: PDA detector (Waters 2996, Waters, MA, USA) wavelength: 210–400 nm, column: Inert Sustain C18 5 μm, 4.6 x 250 mm I.D., mobile phase: acetonitrile/0.1 vol% aqueous acetic acid solution = 30/70, flow rate: 1.0 ml/min, temperature in column oven: 40°C [20, 21].

**Determination of antioxidant activity by DPPH scavenging assay**

In 96-well plates, each 100 μl flavonoid sample was appropriately diluted by the addition of 50% ethanol. A DPPH radical solution (concentration: 600 μM), prepared by dissolving the DPPH radical in 100% ethanol in advance, was added in aliquots of 100 μl per well (finally making an volume of 200 μl per well with 75% ethanol concentration). After stirring on a shaking table till sufficient mixing had occurred, the plate was allowed to stand in a dark place for 30 min. The absorbance OD517 at the DPPH absorption wavelength (517 nm) for each well was measured using a multiplate reader (SpectraMax M5, Molecular Devices, CA, USA) [22]. The DPPH radical scavenging activity (%) was calculated by standardizing the difference between the absorbance OD517 of a measurement sample and the absorbance OD517 of a control (without DPPH) with the absorbance OD517 of the control [23, 24].

**Cell toxicity test in short-time drug treatment**

CHO cells cultured semi-confluently beforehand in a 100 mm culture dish were trypsinized. In 24-well plates, 40 000 CHO cells were seeded per well and cultured for one day. After the culturing, the medium was removed by an aspirator and the cells were washed twice with phosphate buffer saline (PBS(−)), and this was followed by the addition of a drug (250, 625, 1250, 2500, 5000 μM) dissolved in PBS(−). The plate was left in the incubator for 30 min. After that, PBS(−) solutions were removed with an aspirator and the cells were washed twice with fresh PBS(−); the cells were then released by trypsin treatment, suspended in αMEM (10% FBS), and the cell number was measured using a Coulter Counter Z1. The cell growth rate was calculated by standardizing the obtained cell count against the cell count obtained without addition of the drug [25].

**Analysis of intracellular ROS induced by ionizing radiation**

CHO cells were put into a 24-well plate (40 000 cells/well) and cultured for one day. After the culturing, the medium was removed by an aspirator and the cells were washed twice with PBS(−), followed by addition of a flavonoid or its composites (250 μM) dissolved in PBS(−) and 200 μl of the reagent for measurement of ROS [2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA); 10 μM] to each well. The plate was stored in a dark place at 37°C for 30 min and then was irradiated with 4 Gy of X-rays. After the irradiation, the intracellular 2′,7′-dichlorodihydrofluorescein (DCF) fluorescence intensity was measured by using a fluorescence plate reader (Spectramax M5, Molecular Devices, CA, USA) at 485 nm excitation/538 nm emission. The obtained DCF fluorescence intensity was standardized with the DCF fluorescence intensity in the cells without irradiation and drug addition to calculate the ROS inhibition rate [26, 27].

**Colony-formation assay**

CHO cells treated with a flavonoid (250 μM) under the same conditions as the ROS analysis described above were irradiated with 0, 2, 4, 6 and 8 Gy of X-rays. After the irradiation, PBS(−) was removed by an aspirator and the cells were washed with PBS(−) again. Then, the cells were released by trypsin treatment and suspended in a medium. Part of the cell suspension was subjected to measurement of the cell count using Coulter Counter Z1, and the cell suspension was diluted to an appropriate concentration according to the X-ray irradiation dose and then put into a 100 mm culture dish together with the medium. After static culture for 10 days, the medium was removed by an aspirator and the cells were washed twice with PBS(−), followed by immobilization of the cells with 100% ethanol. Thereafter, the cells were stained with a crystal violet staining solution, followed by washing with water and drying. The number of colonies (50 or more cells/colony) on the culture dish was measured by visual inspection, and the viability was calculated by comparing the number of colonies with that obtained without irradiation (0 Gy).

**Measurement of intracellular glutathione concentration**

The concentration of reduced glutathione (GSH) in CHO cells was measured by the fluorescence intensity of the GSH conjugate of monochlorobimane (mBCl) [28]. First, CHO cells were put into a 96-well plate (40 000 cells/well) and cultured for one day. After the culturing, the medium was removed by an aspirator and the cells were washed twice with PBS(−), followed by addition of flavonoids (250 μM) dissolved in PBS(−). The plate was allowed to stand in an incubator for 30 min and then the medium was removed by an aspirator and the cells were washed twice with PBS(−). The CHO cells were incubated with mBCl (50 μM) dissolved in PBS(−) for 30 min at 37°C, and the fluorescence intensity was measured with an excitation wavelength at 385 nm and emission wavelength at 485 nm, using a fluorescence plate reader (Spectramax M5, Molecular Devices, CA, USA). The fluorescence intensity obtained was standardized by comparing it with the fluorescence intensity of mBCl conjugate in control cells and thus used to calculate the content of intracellular GSH.

**PARP inhibition assay**

Poly (ADP-ribose) polymerase (PARP) inhibition was measured using the PARP colorimetric assay kit (Trevigen, Gaithersburg, MD) [25, 29]. PARP was incubated in a 96-well microplate with a reaction mixture containing 50 μM β-NAD+ (10% biotinylated β-NAD+, 90% unlabelled β-NAD+), 1 mM 1,4-dithiothreitol and...
1.25 mg/ml nicked DNA. The formation of poly (ADP-ribose) polymers was detected with peroxidase-labeled streptavidin and 3,3′,5,5′-tetramethylbenzidine (Invitrogen, Carlsbad, CA). PARP inhibition was assessed by the addition of aglycones (QUE, HES, NAR) at various dosages to the reaction mixture. PARP activity was calculated by measuring the absorbance at 450 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Statistics
All experiments were performed at least three times independently, and the statistical significance was confirmed by using one-way ANOVA followed by Tukey’s multiple comparisons test with Prism 6 software (GraphPad Software, Inc., CA, US). In all data, P values less than 0.05 were considered to be statistically significant, those with $P < 0.05$ vs control were marked with *, those with $P < 0.01$ vs control were marked with **, those with $P < 0.05$ vs MGR were marked with #, those with $P < 0.01$ vs MGR were marked with ##, those with $P < 0.05$ vs AA were marked with $\$\$ and those with $P < 0.01$ vs AA were marked with $\$$. Each error bar represents the standard error of the mean.

RESULTS
Measurement of water solubility of flavonoid composites
The results of the water solubility measurement of the aglycone and flavonoid composite by HPLC are shown in Fig. 2. The initial concentration of the aglycone was 100 μM, and its concentration was measured over time (Fig. 2A–C). First, in the case of QUE/MGR, the concentration of QUE solubilized with MGR was hardly changed from the initial concentration of 100 μM; however, the concentration of QUE solubilized with 1% DMSO began to decrease at 5 min, and the aggregates were observed in the aqueous solution as the concentration of QUE decreased. Finally, after 1 h, it was almost the same as the concentration of QUE dissolved only with DW (Fig. 2A). Further, as to HES and NAR, it was confirmed that the solubilized state was maintained for ~1 h in the case of HES dissolved with MGR and in the case of NAR dissolved with 1% DMSO or MGR (Fig. 2B and C). On the other hand, we observed that aggregation started with decreasing concentration of HES solubilized with 1% DMSO.

Evaluation of the antioxidant potency of flavonoid composites
As a result of comparative tests with aglycones and MGR alone in the DPPH radical scavenging assay shown in Fig. 3, the descending order of antioxidant activity at 500 μM concentration was as follows; QUE (81%) > MGR (66%) > AA (60%) > HES (18%) > NAR (4%), and it showed a significant difference ($P < 0.05$) from that of AA. MGR in the quercetin family did not show a significant reduction in antioxidant effect due to transglycosylation (Fig. 3). In addition, in the comparison between the flavonoid composite and MGR, the descending order of antioxidant activity at 500 μM concentration was QUE/MGR (74%) > MGR (66%) > HES/MGR (59%) > NAR/MGR (54%), and they showed a significant difference ($P < 0.05$) from that of MGR. QUE/MGR was the highest among all flavonoid composite results, and the total antioxidant activity of the flavonoid composite changed depending on the type of aglycone contained in the flavonoid composite (Fig. 3).

Cell toxicity of flavonoid composites in short-term drug treatment
With short-term drug treatment (30 min), only MGR-treated cells showed no toxicity at up to 5 mM. Among the flavonoid composites, the cell growth rate began to decrease with statistical significance ($P < 0.05$ vs MGR at 625 μM) from the concentration...
of QUE/MGR at a molar average concentration of 625 μM, that is, the concentration at which QUE contained 125 μM. On the other hand, in both HES/MGR and NAR/MGR, the cell growth rate began to decrease with statistical significance (P < 0.01 vs MGR at 2.5 mM) from the concentration containing 2.5 mM in molar average concentration, in other words the concentration containing 500 μM of HES and NAR, respectively. They showed that the flavonoid composite had cytotoxicity depending on the aglycone contained therein, and of these aglycones, QUE has particularly high cytotoxicity (Fig. 4).

**Radioprotective effect of flavonoid composites in colony-formation assay**

In the comparative study of MGR- and drug-untreated cells, the survival rate of MGR-treated cells (using 250 μM MGR) increased with all irradiation doses (2, 4, 6 and 8 Gy), but did not show a significant radioprotective effect (Fig. 6). In addition, in comparative tests of flavonoid composite–treated cells and drug-untreated cells, when cell viability was compared at irradiation doses of 2, 4, 6 and 8 Gy.
8 Gy, cell viability was evaluated in the order of QUE/MGR (250 μM) < NAR/MGR (250 μM) < HES/MGR (250 μM), and HES/MGR and NAR/MGR showed a confirmed radioprotective effect with a statistically significant difference (HES/MGR; P < 0.01, NAR/MGR; P < 0.05) from the drug-untreated control at an irradiation intensity of 6 or 8 Gy. In particular, HES/MGR showed the highest increase in cell viability among the flavonoid composites tested in this study. Also, in a comparative study between MGR alone (250 μM) and the flavonoid composite HES/MGR, there was a statistically significant increase in cell survival (P < 0.01) compared with MGR controls with irradiation doses of 8 Gy. However, for QUE/MGR, there was no statistically significant difference for any of the irradiation doses (Fig. 6).

Influence of flavonoid composites on intracellular GSH concentration
When the change in the intracellular GSH concentration was confirmed by aglycone alone administration, it was shown that QUE decreased the intracellular GSH concentration dependently (54% at 50 μM and 30% at 250 μM) and that there was a statistically significant difference (P < 0.01) from that of the drug-untreated control. However, neither HES (250 μM) nor NAR (250 μM) changed the intracellular GSH concentration. In addition, when comparing MGR with the flavonoid composite, no change in the GSH concentration was observed with MGR controls with irradiation doses of 8 Gy. However, for QUE/MGR, there was no statistically significant difference for any of the irradiation doses (Fig. 6).

Inhibition of PARP activities of aglycones
Comparing PARP inhibitory concentrations of three aglycones (QUE, HES, NAR), QUE had the highest PARP inhibitory effect (50% inhibitory concentration = IC50 = 329 μM). HES (IC50 = 807 μM) and NAR (IC50 = 823 μM) showed a lower PARP inhibitory effect than QUE. In addition, there was almost no difference in PARP inhibitory effect between HES and NAR (Fig. 8).

DISCUSSION
MGR is a transglucosylated flavonoid with excellent water solubility and antioxidant ability by adding glucose with rutin as a raw material. Unlike aglycone QUE, rutin is a very safe flavonoid that does not show mutagenicity, but there is a disadvantage in that the efficiency of incorporation into cells is significantly decreased [24]. MGR is also a very safe flavonoid, but the problem of its poor uptake efficiency into cells is similar to that of rutin. It is important to solve the problem of cellular uptake efficiency of MGR in the study of radioprotective agent using MGR. Therefore, in this study, we aimed to search for more effective radioprotective agents using flavonoid composite utilizing hardly water-soluble flavonoid (aglycone) and solubilizing action of MGR. In other words, in addition to the radiation protection effect of the original MGR, the purpose of this study was to confirm that it acts more effectively as a radioprotective agent by adding the intracellular action of aglycone.
activities that could not be achieved by MGR alone, by changing the aglycone to be combined.

In addition, as a result of intracellular ROS scavenging assay, the flavonoid composite showed intracellular ROS scavenging ability depending on the antioxidant capacity of the aglycone contained. QUE/MGR and HES/MGR showed statistically significant ROS scavenging activity against irradiated control. In particular, QUE/MGR showed higher intracellular ROS scavenging ability than other flavonoid composites due to the QUE contained therein (Fig. 4). This result means that the aglycone is taken into the cell from the flavonoid composite and exerts as a ROS scavenger in cells. In addition, from previous studies showing the effect of improving the bioabsorption efficiency of poorly water-soluble substances indicated in mouse oral intake tests, MGR has been demonstrated as a carrier for drug delivery system (DDS) that promotes the absorption of poorly water-soluble substances in vivo and intracellularly [10, 13].

Next, in order to investigate the possibility of a flavonoid composite as a radioprotective agent, a colony assay test was carried out. The concentration of the administered drug (250 μM) was set to the concentration at which cytotoxicity of non-irradiated cells was not observed in the cytotoxicity test described above. As a result, among the three flavonoid composites, HES/MGR and NAR/MGR showed radioprotective effect against drug non-administered control, and furthermore HES/MGR indicated significant radioprotective effect was against MGR alone. From the results of this test, it was found that HES was the most suitable among three types of aglycone combined with MGR. On the other hand, QUE/MGR showed high antioxidant effect, showed a strong removal effect against radiation induced ROS, and high radiation protection effect was expected, but no significant radioprotection was seen in this test result. These results indicate that when designing an excellent flavonoid composite as a radioprotective agent, the selection of the aglycone to be used will consider not only the antioxidant effect such as having high ROS scavenging activity but also other noteworthy functionalities.

Finally, in order to ascertain the cause of the weak radioprotective effect indicated by QUE/MGR, it was confirmed the influence on the intracellular GSH concentration by the flavonoid composite. As a result, changes in intracellular GSH concentrations were measured in QUE alone or in QUE/MGR containing QUE, but for MGR, HES, NAR alone and flavonoid composites containing HES or NAR, there was no change in GSH concentration. This shows that QUE is oxidized in the cell, becomes QUE oxide, and binds to intracellular glutathione, whereby the GSH concentration decreases. As a cause of this, previous studies have confirmed that QUE oxide binds to the sulfhydryl group (SH group) of glutathione [31]. Reduction of intracellular GSH by QUE not only lowers the resistance of intracellular oxidative stress but also promotes the decline of cell recovery function utilizing glutathione conjugation that is one of the main functions of GSH [32]. Furthermore, it is considered that the binding of the QUE oxidized to the SH group in such cells causes not only glutathione but also inhibition of the activity of the protein regulated by disulfide bonds [33]. In this study, we confirmed that QUE had a stronger PARP activity inhibitory effect than HES and NAR. Inhibition of PARP activity adversely affects the repair function of DNA damaged by radiation.

First, a solubility test to solubilize a poorly water-soluble aglycone with MGR was performed by comparing with DMSO. As we used a simple dissolution method only by adding the sample to PBS (−) and stirring with a vortex mixer for 1 minute in our experiment, in the case of aglycone (QUE, HES) with low solubility and high crystallinity, dissolution in aqueous solution of 1% DMSO was insufficient and aggregation started. On the other hand, it was revealed that MGR solubilized not only QUE with low water solubility but also other hardly water-soluble flavonoids (HES, NAR). These experimental results indicated that MGR was a flavonoid-based solubilizer suitable not only for aglycone QUE of MGR but also for two types of aglycone (HES, NAR) used in this study.

Furthermore, in the DPPH radical scavenging assay, the antioxidant activity of the flavonoid composite varied depending on the type of aglycone combined. This indicates that by combining aglycone and MGR, the antioxidant activity of both substances was maintained and was not inhibited by mixing with each other and this may results in the potential mechanisms for radioprotection.

The effect of aglycone cellular uptake by flavonoid composite was confirmed by cytotoxicity test by drug treatment for a short time (30 min). The reason why the drug treatment time was set to 30 min was based on the previous studies that the uptake rate of aglycone alone reached within the nucleus after permeating through the cell membrane within 30 min [30]. In the cytotoxicity test in this study, MGR alone showed no significant cytotoxicity at any concentration, that is, MGR had a low cell uptake effect and was a favorable result as an aglycone carrier. Next, the result of the flavonoid composite, the most toxic was QUE/MGR, which showed clear cytotoxicity at a concentration of 1.25 mM or more, that is, a QUE concentration of 250 μM or more. Also, for HES/MGR and NAR/MGR, since cytotoxicity is lower than QUE/MGR, it can be said that HES and NAR have less intracellular effects than QUE, that is, cytotoxicity is low. From this fact, it was shown that flavonoid composites were able to generate biological

Fig. 8. Comparison of PARP activity inhibitory effect by three kinds of aglycones (QUE, HES, NAR). Error bars indicate standard errors of the means. Three independent experiments were carried out.
In conclusion, we designed three novel flavonoid composites (QUE/MGR, HES/MGR, NAR/MGR) and evaluated their radioprotective effects compared with that of MGR alone administration. QUE/MGR and HES/MGR were expected to have a radioprotective effect by suppressing the rise of intracellular ROS resulting from irradiation, as compared with MGR alone. By the colony-assay test, HES/MGR was confirmed to have a radioprotective effect; however, QUE/MGR was not confirmed to have remarkable radioprotective effect. This suggested that QUE introduced into cells in the form of QUE/MGR acts as an intracellular antioxidant; however, it becomes QUE oxide and binds to the SH group in cells, whereby the intracellular GSH concentration decreases and the PARP activity is inhibited. Thus, since QUE has both antioxidant and pro-oxidant functions in cells, it is expected that a radioprotective effect of QUE will not be clearly confirmed. In other words, QUE is a strong antioxidant, but is highly cytotoxic and therefore undesirable to use in the form of a flavonoid composite as a radioprotector. In contrast, HES has a lower antioxidant activity than QUE, but HES suppresses the increase in intracellular ROS caused by irradiation and its oxide is not a factor that suppresses the radioprotective effect; thus, as a radioprotector it is a preferred aglycone in combination with MGR in the form of a flavonoid composite. We concluded that HES/MGR was the most desirable radioprotector among three types of flavonoid composite examined in this study.

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