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방사선융합 생물분자 구조변환 및 상승작용 이용 연구

Structural Change of Biomolecules and Application of Synergistic Interaction by Radiation

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한 국 원 자 력 연 구 원

제 출 문

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요 약 문

Ⅰ. 제 목

방사선융합 생물분자 구조변환 및 상승작용 이용 연구

Ⅱ. 연구개발의 목적 및 필요성

한국원자력연구원 정읍 방사선과학연구소는 방사선 연구개발에 관한 국가 전문기관이다. 지금까지 방사선을 단순 조사 위주로 이용해 오던 평면적 기술에서 탈피하여 방사선 기술을 기반으로 하여 생명과학기술, 환경기술, 정보기술, 문화기술 등을 복합적으로 활용하여 한 차원 높은 방사선 융·복합 기술을 개발할 필요가 있다. 따라서 본 연구는 방사선 융합 기술을 이용하여 생물분자의 과학적, 산업적 이용가치를 창출하는 한편, 방사선의 긍정적 이용 효율을 극대화하기 위한 체계 정립에 역점을 두고 수행되었다.

Ⅲ. 연구개발의 내용 및 범위

본 연구개발에서는 다음과 같은 주요 내용과 범위가 연구개발에 포함되었다.

○ 단백질 또는 펩티드의 기능적 특성 증진을 위한 방사선 구조변환 기술 개발
○ 다당류의 물성개선 및 저분자화를 위한 방사선 구조변환 기술 개발
○ 생물유래 복합추출물 및 이차대사산물의 이화학적 특성 및 기능성 증진 기술 개발
○ 방사선 준위 및 복합처리 조건에 따른 생물영향 특성 연구
○ 방사선과 다요인 복합작용의 규칙성 도출 및 상승작용 이론모델 구축

Ⅳ. 연구개발 결과

1. 방사선 구조변환 기술이용 실크 피브로인 단백질 개발

누에에서 생산되는 실크는 섬유상 단백질인 피브로인과 이들을 감싸고 있는 구형 단백질인 세리신으로 구성되어 있으며 피브로인은 견사의 전체 중량의 70 ~ 80%정도이며 세리신은 20 ~ 30%정도를 이루고 있다. 실크단백질 중 피브로인과 세리신은 각각 10 ~ 200 kDa과 65 ~ 400 kDa 정도이다. 정련단계를 거쳐 세리신을 제거한 피브로인은 수천년 동안 의류용 실크로 널리 이용되어 왔으며, 급속한 경제성장과 산업화로 인해 최근

입는 누에고치에서 식용, 미용의 실크 단백질의 생산이 가능하게 되었다. 또한 이의 생리활성이 밝혀지며 식품, 화장품, 의약품 등의 산업적 이용방안 연구가 활발히 진행되고 있다. 한편 이러한 많은 연구에도 불구하고 아직까지 방사선 조사기술을 이용하여 실크 단백질의 구조적인 변화와 생리활성에 관한 연구는 미흡한 실정이다. 방사선은 강력한 에너지원으로써 물질을 변형시키거나, 파괴시킬 수 있으며 현재 멸균을 위한 목적으로 많이 사용되고 있는 추세이며 단백질에 방사선 조사를 했을 경우 oxygen radical에 의한 화학적인 변화는 fragmentation, cross-linking, aggregation 그리고 oxidation이 있다. 그러므로 본 연구에서는 소재개발을 위한 기초연구로써 방사선에 대한 실크 단백질 (피브로인)의 영향과 조사된 실크 단백질의 생리활성에 관하여 연구하였다. 본 연구에서 피브로인의 구조변화 측정은 GPC, SDS-PAGE, UV, CD에 의해서 연구되었다. 이러한 실험의 결과들은 방사선 선량이 증가함에 따라 단백질 분자량이 증가 하였고 단백질 2차 구조 변화의 평가에서 10 kGy까지는 α-helix구조가 증가하는 경향을 보이다가 50 kGy부터 다시 감소하는 경향 나타났고 이와는 반대로 β-sheet, β-turn, 그리고 random coil은 10 kGy까지는 감소하다가 50 kGy부터는 다시 증가함을 확인 할 수 있었다. 생리활성의 측면에서는 볼 때 방사선 조사된 피브로인은 방사선 조사선량이 증가함에 따라 항산화 활성과 미백활성 효과를 확인 할 수 있었다. 방사선 조사된 피브로인의 면역 활성 평가를 위해 암세포와 정상세포에 의한 세포독성 평가실험에서 방사선 조사된 피브로인은 암세포에 대한 독성이 있었고, 면역세포에 대한 세포독성 평가로서 피브로인은 면역세포를 활성 시켰다. 동물실험에서 mouse의 tumor growth inhibition의 활성 평가와 NK cell, T-cell, macrophage, cytokine (IL-6, 2, 12, IFN-ɣ, TNF-α)들의 활성을 평가한 결과, 방사선 조사된 실크 피브로인에 의한 여러 면역반응의 증가가 mouse의 tumor growth의 성장을 억제 하였다. 이러한 결과들로부터 방사선 조사된 피브로인은 식품과 화장품 산업뿐만 아니라 의학 산업에도 가치 있는 산출물로서 잠정적인 candidate가 될 수 있으며, 생리활성 및 면역 활성의 원인규명을 위한 더 많은 연구 수행이 필요하다고 사료된다.

2. 물성(점도/용해도) 및 기능성 증진된 저분자 베타글루칸 개발

현재 베타글루칸은 미국에서 차세대 기능성 건강식품으로 인정되어 높은 판매량을 보이고 있으며 1983년 미국 FDA가 규정하는 일반 안전기준(GRAS 규격 title 21, vol 3)에 기재되어 있다. 그리고 베타글루칸은 겔화 및 수용액에서 점도상승 능력 때문에 소규모 식품산업 등에서 사용하고 있으나, 공정상에 문제를 야기하는 높은 점도와 낮은 용해도로 인해 기능성 식품의 첨가제로 어려움을 가지고 있다. 따라서, 본 연구는 감마선 조

사에 의한 β-glucan의 분자량 감소, 점성 감소, 용해도 증진과 같은 이화학적인 특성 변화 및 면역기능 향상 효과 측면에서 살펴보고자 하였다.

먼저 분말 상태인 β-glucan을 증류수에 용해시킨 후 β-glucan 용액을 0, 10, 30, 50 kGy로 감마선 조사를 하였다. 감마선 조사된 β-glucan 용액을 가지고 점도, 용해도 측정과 미생물을 측정해 본 결과 점도는 감소하였으며, 용해도는 증가하였고, 미생물은 유의적으로 감소하였으며 50 kGy에서는 모든 미생물이 검출되지 않았다. 그리고 방사선에 의한 β-glucan의 구조를 분석하기 위하여 gel permeation chromatography (GPC), UV-VIS absorption sprectroscopy, fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM)을 통해 감마선 조사된 β-glucan의 구조적 특성을 측정하였다. 그 결과 β-glucan 내의 다른 기능성들은 변화가 없었으며, β-glucan의 주요사슬인 glycosidic bonds가 끊어지면서 저분자화된 것을 확인 할 수 있었다. 이러한 이화학적 변화에 따른 면역 기능의 효과를 확인하기 위하여 β-glucan의 주요 효능으로 알려진 면역력 강화 효과와 cholesterol (LDL) 감소 효과를 cell proliferation, cytokines assay, blood cholesterol (HDL & LDL) assay 와 같은 실험방법으로 평가하였다. 실험결과 *in vitro* 와 *in vivo* 에서 모두 spleen cell profilation의 증가, cytokine 방출량의 증가 등의 면역력 강화 효과가 나타났으며, *in vivo* 에서는 7일 간의 경구 투여만으로도 cholesterol (LDL)이 감소하는 결과를 확인할 수 있었다.

따라서 본 연구에서 밝혀진 감마선 조사에 의한 베타글루칸의 물성 개선으로 식품산업의 이용분야가 확장 될 것이고 면역력이 약한 노약자, 어린이, 환자들에게 더욱 효과적이고 안전한 식품 첨가제로 사용에 기여할 것으로 사료된다.

3. 차가버섯 폴리페놀의 색상 개선 및 항산화활성 증진기술 개발

본 연구에서는 감마선 조사된 차가버섯 추출물의 색도와 항산화능에 관하여 조사하였다. 차가버섯 추출물을 10 ㎎/㎖의 농도로 물에 용해시킨 후 0, 3, 5, 7 10 kGy 흡수방사선으로 조사하였고, 이때 색도, 항산화능, 총 폴리페놀의 함량을 각각 측정하였다. 색도의 경우 감마선 조사선량에 의존적으로 명도 (lightness), 황색도 (yellownwss)가 증가하였으며, 반면에 적색도 (redness)는 감소하였다. 감마선 조사된 차가버섯 추출물의 항산화능 평가는 DPPH 라디칼 소거능, UWLA (ultraweak chemiluminescence assay)를 이용한 초산소 및 수나 자유라디칼 소거능과, 철 이온을 이용한 환원력 측정, 지방 산화 억제능 측정을 수행하였으며, 조사선량에 의존적으로 항산화능도 증가 하는 결과를 관찰 할 수 있었다. 또한 각각의 조사선량에 따라 폴리페놀의 함량을 측정한 결과, 마찬가지로 조사선량에 따라 증가하는 경향을

나타내었다. 이러한 결과로 미루어 볼 때 감마선의 조사는 차가버섯 추출물의 색도 및 항산화능 개선에 매우 효과적으로 작용함을 알 수 있었다.

4. 방사선 생물영향 평가 및 검출기법 개발 : 방사선 및 염화수은(Ⅱ)이 어류간암세포 사멸에 미치는 상승작용 평가

모든 생물체는 환경 내 존재하는 화학물질과 물리적, 화학적, 사회적 요인 등에서부터 여러 요인의 특성에 따른 복합작용까지 다양한 유해인자의 영향을 받으면서 살아가고 있다. 복합작용 중에서도 둘 이상의 요인에 대해서 생물에 나타나는 영향이 단독영향의 합보다 클 경우, 상승작용이라고 하며, 다중 요인의 복합작용을 연구하기 위해서는 자극에 보다 민감하게 반응하는 생물을 이용하여 이들의 생체내 손상 정도를 평가 및 예측하는 연구가 필요하다. 이에 따라, 본 연구에서는 수환경 오염으로부터 직접 노출되는 생물인 어류의 세포 (PLHC-1 세포주)를 대상으로 이온화 방사선과 수은에 동시에 노출되었을 경우, 그 손상을 생존율 변화를 통해 관찰하고자 하였다. 방사선과 수은의 단독처리군의 경우, 생존율에 따른 LD_{50} (24 hr) 값은 각각 298.03 Gy, 164.12 uM로 산출되었다. 방사선과 수은의 복합처리에 의한 실험 결과에서는 두 가지 요인의 자극 강도가 강해짐에 따라 생존율이 현저하게 감소되었으며, 각 요인의 상호작용에 의한 영향이 이론적인 단순가산값 보다 높게 나타나 상승작용으로 확인되었다. 이러한 결과는 방사선과 염화수은(Ⅱ)에 복합처리된 세포의 생존 변화로써 각 물질의 상승작용을 확인할 수 있음은 물론, 각 요인의 방사선 자극에 대하여 PLHC-1 세포주를 이용한 생태영향평가의 가능성을 확인할 수 있었다. 이와 더불어, 향후 다양한 생체지표를 활용함으로써 방사선 생태영향평가의 기초이론 정립은 물론 복합작용의 정량화할 수 있을 것으로 기대된다.

5. 방사선 생물영향 평가 및 검출기법 개발 : 이온화 방사선과 염화수은 복합처리에 따른 세포사멸 및 유전자 발현

*Saccharomyces cerevisiae*는 인간세포에 있는 생물학적 과정을 연구하는 이상적인 모델이다. 또한, 상업적인 생물학 실험을 위해 사용된다. 모든 호기성 생물들은 성장하면서 분자형태 산소의 부분적 감소인 활성산소종에 의하여 산화적 스트레스를 받는다. 산화적 스트레스에 의해 세포의 구성물질인 단백질, 지질과 DNA의 손상을 일으킨다. 따라서, 서로 다른 많은 생물들의 세포에서 활성산소종에 대항하는 방어기작을 일으킨다. 중금속 및 이온화 방사선 등과 같아 활성산소종의 생성 유발에

대하여 저항하는 유전자의 존재가 알려져 있다. 방사선과 유해화학물질의 복합처리에 따른 산화적 스트레스에 의한 생물영향을 평가하고 검출하는 기술을 개발하기 위한 연구의 일환으로 효모세포를 이용한 복합처리 실험을 수행하였다. 먼저, 이온화 방사선, 염화수은 (Ⅱ) 그리고 이온화방사선과 염화수은 (Ⅱ) 복합처리에 따른 세포의 생존율을 알아보았다. 아무런 처리를 하지 않은 세포를 대조군으로 두고, 100, 400, 800, 1200 Gy hr^{-1} 의 4가지 선량률에 최종흡수선량을 각각 400 Gy, 800 Gy로 조사하였다. 염화수은 (Ⅱ)을 0.1-0.9 mM 범위로 처리하여 주었으며, 선량률이 400, 800 Gy hr^{-1} 조건으로 이온화 방사선과 염화수은 (Ⅱ)을 복합 처리하였다. 실험은 3차례 수행하였으며 3회 실험값의 평균을 이용하여 세포의 생존율을 구하였다. 동일한 흡수선량 조건에서 세포의 생존율은 방사선 선량률에 따라 다르게 나타났다. 선량률이 높을수록 세포의 생존율은 낮았고, 특정 선량률 이상의 범위에서 세포는 거의 사멸하게 된다. 복합처리에 따른 세포의 생존율 또한 감소하였다. 염화수은 (Ⅱ) 처리를 통해 중금속 저항 유전자를 발현시켰고, 이온화 방사선 처리를 통하여 방사선 저항 유전자들을 발현시켰다. Real-time PCR을 통하여 YCF1과 YAP1 유전자의 발현을 조사하였다. 두 유전자는 수은 농도에 의해 발현되었고, 낮은 선량률의 높은 흡수선량 조건에서 발현되었다. 그러나, 0.2 mM 염화수은 (Ⅱ) 처리 조건에서는 다르게 발현하였다. 두 유전자는 높은 선량률의 낮은 흡수선량조건에서 더 발현되었다. 0.1 ~ 0.2 mM 염화수은 (Ⅱ) 처리 조건에서 세포의 생존율은 다른 농도조건에 비하여 증가하였다. 결과적으로 세포생존과 관련된 산화적 스트레스 저항유전자의 발현은 스트레스가 클수록 증가하는 것을 알 수 있었다. 염화수은 (Ⅱ)과 방사선을 복합 처리하였을 때 스트레스의 단독 처리했을 때보다 유전자의 발현이 증가되는 상승효과를 나타내었다.

6. 방사선과 복합처리 조건에 따른 상승작용 이론 수립 및 검증

이온화 방사선을 또 다른 물리·화학적 요인과 복합처리함으로써 방사선의 전반적인 생물학적 영향을 증대시킬 수 있는데 이러한 현상을 다음과 같은 두 가지 측면에서 설명할 수 있다. 첫째, 두 요인의 복합처리에 따라 세포 손상수복 능력이 현저히 저해되었다고 볼 수 있다. 둘째, 각각의 요인에 의해 유발된 준손상 간의 상호작용에 의해 부가적 치사손상이 유발됨으로써 상승작용이 나타난다고 해석할 수 있다. 이때 각개 요인에 의한 초기의 준손상은 생물학적 효과를 나타내지 못하는 잠재 손상으로 생각할 수 있다. 이와 같은 해석에 근거한 이론 모델을 수립하였다. 수립된 이론을 이용하여 상승작용과 방사선량 및 선량률 간의 의존성을 분석할 수 있을 뿐 아니라 최대상승작용의 정도와 조건을 예측해 볼 수 있다. 본 항의 연구에서는 박테리오파지, 세균포자, 효모 및 포유동

물 세포에 대한 실험결과와 수립된 이론을 비교·검증함으로써 다음과 같은 결과를 얻었다. (1) 방사선과 고온을 복합처리할 경우에 있어, 선량률이 일정할 때는 특정 온도범위 내에서만 상승작용이 나타난다. (2) 선량률이 감소할 때는 최대상승작용이 나타나는 온도 또한 감소한다. 처리 온도가 일정할 경우, 상승작용을 유발하는 특정 선량률 범위가 존재한다. (3) 복합처리 후 세포 손상수복이 저해되는 것은 단일요인 처리에 비해 비가역 손상이 증가하기 때문이다. 효모세포에 있어서 손상수복의 확률은 온도의존성을 갖지 않는다. 화학물질 처리에 의한 손상수복 저해는 수복기작 자체의 손상 때문에 일어나는 것이 아니라 세포의 비가역 손상이 증가하기 때문이란 사실도 밝혀졌다.

V. 연구결과의 활용계획 및 건의사항

국가 방사선 핵심연구시설을 활용하여 대형국책 연구사업의 원활한 수행을 위한 기초기반기술을 제공할 수 있으므로 향후 새로운 연구과제 도출 및 수행을 위한 동기부여 및 기술기반 제공이 가능할 것으로 전망된다. 방사선과 기존 기술을 병합한 RFT 기술의 적용을 통하여 생물분자의 구조를 변환함으로써 물리적, 화학적, 생물학적 특성을 증진시켜 과학산업적 가치를 창출할 수 있을 것이다. 한편, 이온화 방사선과 기타 요인의 복합 처리 조건별 생물영향 평가 기술을 수립하고 이온화 방사선과 물리·화학적 요인을 복합처리할 때 나타나는 상승작용을 해석하고 최대 상승작용 조건 등을 사전 예측하는 이론을 정립함으로써 방사선의 긍정적 이용효율을 극대화하기 위한 기술로 발전시킬 것이다. 획득한 연구결과를 더욱 발전시키고 개선하여 방사선 이용기술의 고도화를 위한 연구개발 및 응용분야의 기반 을 확립함을 물론 기초과학, 산업, 의학 등 다양한 분야에 방사선을 효과적으로 활용하기 위한 기술근거 제공 효과를 거둘 수 있다.

SUMMARY

Ⅰ. Title of Project

Structural Change of Biomolecules and Application of Synergistic Interaction of Radiation

Ⅱ. Objectives and Necessity of Research

As Advanced Radiation Technology Institute plays a key role of a national research organization for radiation R&D, its necessity of fundamental research on radiation applications has arisen. And thus the objective of this study is to develop fundamentals for radiation applications based on the existing radiation technology, to develop new functional materials through structural changes of biomolecules, and to establish technical basis for enhancing efficacy of radiation utilization by studying the simultaneous application of ionizing radiation with another factor.

III. Work Scopes of Research

The research consisted of the following work scopes.

- ○ Effect of gamma irradiation on the structural and physiological properties of silk fibroin
- ○ Improvement of the physicochemical and immunomodulatory property of β-glucan by gamma irradiation
- ○ Improvement of color and antioxidant properties of Chaga mushroom (*Inonotus obliquus*) extract by gamma irradiation
- ○ Biological effects of the combined treatment of ionizing radiation and another factor: Synergistic effects of ionizing radiation and mercury chloride(Ⅱ) on death of fish hepatoma cells
- ○ Biological effects of the combined treatment of ionizing radiation and another factor:
  Cell survival and gene expressions after treatment of mercury chloride (Ⅱ) and ionizing radiation
- ○ Theoretical Conception of Synergistic Interactions and their regularities

IV. Results of Research

1. Effect of Gamma Irradiation on the Structural and Physiological Properties of Silk Fibroin

Silk derived from silkworm *Bombyx mori* is a natural protein which is mainly made of sericin and fibroin proteins. Silk-based materials have been used in the various fields of industries such as functional food, cosmetics and biomedical material. The present study was to investigate the structural and physiological changes of fibroin by gamma irradiation and also include the immune functions in *in vitro* and *in vivo* model using irradiated fibroin, in order to explore the feasibility of industrialization of fibroin by using gamma irradiation.

The average molecular weights of irradiated fibroin were measured by gel permeation chromatography (GPC), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was measured to determine the pattern of molecular weight. The results of GPC and SDS-PAGE showed that the molecular weight of fibroin were increased as the increase of radiation dose. The circular dichroism (CD) data for the determination of secondary structure showed that the fibroin, the content of α-helix increased at 5 to 10 kGy and then decreased at 50 to 200 kGy, while content of the β-sheet, β-turn, and random coil decreased at 5 to 10 kGy and then increased at 50 to 200 kGy. The UV absorption spectra showed that the conformation of an internal aromatic amino acid was disrupted and was exposed to the external environment. Antioxidant effect of the radical scavenging ability of gamma irradiated fibroin had optimal value at 5 kGy with DPPH assay. But, the tyrosinase inhibitory effect of gamma irradiated fibroin increased depending on absorbed dose. Inhibitory effect of melanogenesis by gamma irradiated fibroin was increased as the radiation dose was increased. Immunological activities of gamma irradiated fibroin were carried out with *in vitro* and *in vivo* experiments. The cytotoxic effect of the gamma irradiated fibroin in cancer cell line such as B16BL6, AGS and HT-29, have shown that the cytotoxicity of fibroin was increased depending upon irradiation dose. In the test of macrophage and spleen cell proliferation, the immune activities of fibroin were increased by gamma irradiation. Based on the results of *in vitro* study, the most effective dose (150 kGy) for fibroin irradiation was selected and *in vivo* study with

C57BL6 and BALB/c mouse was conducted at that dose.

In experimental tumor using B16BL6 melanoma cells, the gamma irradiated fibroin (150 kGy) significantly showed the tumor growth inhibitory effects higher than those of untreated and 50 kGy fibroin. Assay of cell populations such as NK cell, $CD4^+$ / $CD8^+$ T-cell, macrophage and cytokines (IL-6, 2, 12, IFN-γ and TNF-α), the gamma irradiated fibroin significantly inhibited tumor growth via activation of NK cells population, T lymphocyte, macrophage and cytokines.

From these results, it could be concluded that the gamma irradiated silk fibroin might be a potential candidate as a value-added product in food, cosmetic and medical industry.

2. Improvement of the Physicochemical and Immunomodulatory Property of β-glucan by Gamma-irradiation

β-glucan is a scientifically established biological defense modifier (BDM) that nutritionally potentiates and modulates the immune response. As a supplement, after swallowing orally, β-glucan is ingested primarily through macrophage and dendritic immune cells by immune response potentiation and modulation. For many years glucans have been investigated for these immune enhancing properties, particularly their ability to activate macrophage and NK-Cells in turn, the T-Cells, and B-Cells including selected cytokines and complement system. In the present study we have examined the physicochemical properties of irradiated and non-irradiated β-glucan using Fourier Transform Infrared Spectroscopy (FTIR), Gel Permeation Chromatography (GPC), and UV spectroscopy. And we explored the importance of irradiation technology on high molecular weight β-glucan to obtain low molecular weight β-glucan without any major changes in biological function. Irradiated β-glucan showed some meaningful improvement in the viscosity, solubility and immunodulatory property. *In vitro* DPPH assay and microbial test were also carried out which showed irradiated β-glucan (low molecular weight) has some improvement *in vitro* antioxidant nature and have potent role to defend microorganisms over native β-glucan molecule. We have also attempted a study on low molecular weight β-glucan (*in vitro* and *in vivo*) of non-irradiated and irradiated (10, 30 and 50 kGy) β-glucan on spleen cells. *In vivo* and *in vitro* investigation using irradiated β-glucan

displayed profound effect on spleen cell proliferation (MTT assy) in dose dependent fashion compared to non-irradiated β-glucan. As cytokine played a major role in immunodulatory function we assessed two well know Th1- associated cytokines like IL-2 and IFN-gamma by ELISA method, where irradiated β-glucan found to have prominent role in stimulating immune response. We has also scrutinized its immunomodulatory effect in animal model (Balb/c mice) by administration of irradiated and non-irradiated β-glucan for a period of 7 days. Many studies have reported that β-glucan plays a vital role in lowering bad (low-density lipoprotein, LDL) cholesterol levels. As a primary approach we examined the effect of irradiated β-glucan on bad and good cholesterol in normal mice. Cholesterol (LDL) lowering effect of irradiated β-glucan was relatively better than non-irradiated β-glucan and slight improvement in the high-density lipoprotein (HDL) levels confirmed its importance in cholesterol lowering ability over high molecular weight β-glucan. Based on the above mentioned studies we hopes that irradiation technology can be reinforced for high molecular weight functional foods, so as to improve their physicochemical, digestibility and immunomodulating properties. Thus β-glucan after irradiation can have an enhanced physiological activities with amplified immunostimulatory properties. Irradiation of this natural source may add values and commercial availability to the cometic and pharmaceutical industry, as well as the food industry.

3. Improvement of Color and Antioxidant Properties of Chaga Mushroom (*Inonotus obliquus*) Extract by Gamma Irradiation

The objective of this study was to evaluate the effect of ionizing radiation on color and antioxidative properties of Chaga mushroom (*Inonotus obliquus*) extract (CME). CME (10 ㎎/㎖) was gamma-irradiated at 0, 3, 5, 7, and 10 kGy, and color, antioxidant activity, and total phenolic compound levels of CME were then determined. The lightness and yellowness were increased ($p < 0.05$), and the redness was decreased ($p < 0.05$) as irradiation dose increased. The antioxidant parameters such as the DPPH, superoxide and hydroxyl radical scavenging activities, ferric reducing/antioxidant power, and the inhibition activities of a lipid peroxidation increased as the irradiation dose increased. Also, the total phenolic compound levels

of CME increased ($p < 0.05$) by gamma irradiation. These results suggest that gamma irradiation could be considered as a mean for an improvement of antioxidant properties and the color of CME.

4. Synergistic Effects of Ionizing Radiation and Mercury Chloride(Ⅱ) on Death of Fish Hepatoma Cells

All organisms receive exposure to harmful factors present in the environmental. The combined action of various chemicals is a distinguishing feature of modern life. An interaction between two chemicals is considered as synergistic when the effect produced is greater than the sum of the two single responses. The biological effects due to the combined action of ionizing radiation with the other factor are hard to estimate and predict in advanced. In the current study, we investigated the synergistic effects between ionizing and $HgCl_2$ using fish hepatoma cells (PLHC-1 cells). It was a quite surprising that the results showed a dramatic decrease of cell viability in simultaneous treatment of PLHC-1 cells with ionizing radiation and $HgCl_2$, comparing with neither of them have any cytotoxic effect when treated alone. The cytotoxicity of ionizing radiation was enhanced in the presence of $HgCl_2$. The synergistic effects observed following exposure to ionizing with $HgCl_2$ tested in this study using PLHC-1 cells. The synergistic interaction of radiation and chemical factors was suggested for quantitative prediction of irreversibly damaged cells after combined exposure. Analysis of the extent of synergistic interaction enables to make quantitative estimation of irreversibly damaged cells after the combined exposure. The present study suggests that PLHC-1 cells can serve as rapid screening tools for detecting the toxicity of harmful factors.

5. Cell Survival and Gene Expressions after Treatment of Mercury Chloride (Ⅱ) and Ionizing Radiation

Saccharomyces cerevisiae is an ideal model organism for deducing biological processes in human cells. It is also used for commercial biological experiments. It is known to have some genes for resistance to heavy metals and ionizing radiation (IR). In this work, cell viability was investigated in yeasts treated with IR, $HgCl_2$,

and IR combined with $HgCl_2$. Non-treated cells were used as a control group. Four different dose rates, 100, 400, 800 and 1200 Gy hr^{-1}, were applied to obtain a total dose of 400 and 800 Gy, respectively. Treatment of $HgCl_2$ was implemented in the concentration range from 0.1 to 0.9 mM. Dose rates of 400 and 800 Gy hr^{-1} were used for the treatment of IR combined with $HgCl_2$. Experiments were performed three times and the cell survival rates were calculated from an average of the three experimental data. When the cells were irradiated with the same total dose, there was a difference in the cell survival rates depending on the dose rate. The higher the dose rate was, the lower the cell viability was. At a dose rate above a certain level, the cell viability came down to zero. The combined treatment decreased the cell survival rate, as well. Metal resistance genes were expressed in the cells treated with $HgCl_2$. In a similar way, irradiation also triggered the expression of some radiation resistance genes. As investigated by the Real-time PCR, *YCF* and *YAP* genes were induced consecutively with the $HgCl_2$ concentration, and also with a higher total dose under a lower dose rate condition. These two genes were, however, expressed differently under the 0.2 mM $HgCl_2$ treatment condition. In the cells treated with 0.1~0.2 mM $HgCl_2$, the cell survival rate was higher than with any other concentrations. The results demonstrated that the higher dose induced more expression of oxidative stress resistance genes related to cell survival mechanism. Combined treatment of radiation with mercury chloride resulted in synergistic effects leading to a higher expression of the genes than treatment of a single stressor alone.

6. Theoretical Conception of Synergistic Interactions and their Regularities

An increase in the overall biological effect under the combined action of ionizing radiation with another inactivating agent can be explained in two ways. One is the supposition that synergism may attribute to a reduced cellular capacity of damage repair after the combined action. The other is the hypothesis that synergism may be related to an additional lethal or potentially lethal damage that arises from the interaction of sublesions induced by both agents. These sublesions are considered to be ineffective when each agent is applied separately. Based on this hypothesis, a simple mathematical model was established. The model can predict the greatest value of the synergistic effect, and the dependence of synergy on the intensity of agents

applied, as well. This paper deals with the model validation and the peculiarity of simultaneous action of various factors with radiation on biological systems such as bacteriophage, bacterial spores, yeast and mammalian cells. The common rules of the synergism are as follows. (1) For any constant rate of exposure, the synergy can be observed only within a certain temperature range. The temperature range which synergistically increases the effects of radiation is shifted to the lower temperature for thermosensitive objects. Inside this range, there is a specific temperature that maximizes the synergistic effect. (2) A decrease in the exposure rate results in a decrease of this specific temperature to achieve the greatest synergy and vice versa. For a constant temperature at which the irradiation occurs, synergy can be observed within a certain dose rate range. Inside this range an optimal intensity of the physical agent may be indicated, which maximizes the synergy. As the exposure temperature reduces, the optimal intensity decreases and vice versa. (3) The recovery rate after combined action is decelerated due to an increased number of irreversible damages. The probability of recovery is independent of the exposure temperature for yeast cells irradiated with ionizing or UV radiation. Chemical inhibitors of cell recovery act through the formation of irreversible damage but not *via* damaging the recovery process itself.

V. Plan for Use of the R&D Results

It is expected that motivation and basic technologies for the future R&D plans can be provided from the results of this research. Application of radiation technology together with the existing technologies to enhance the physical, chemical, biological characteristics through structural changes of biomolecules will exert a favorable influence on the creation of *de novo* scientific and industrial values. A theoretical model for the combined action of ionizing radiation with another factor can make it possible to predict *a prior* the maximum value of synergistic interaction and the conditions for it. Furthermore, the results of this study give a clues for establishment of fundamental theories associated with positive efficacy of radiation applications.

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제 1 장 서 론

방사선 연구개발의 핵심 연구시설인 한국원자력연구원 정읍 방사선과학연구소는 국가 방사선 이용기술을 효과적으로 발전시키고 학계, 산업계 등 관련 분야에서 필요로 하는 핵심 기반기술 개발은 물론 산업화에 필요한 응용기술 개발을 사명으로 삼고 있다. 세계적으로 생명과학산업이 주력산업으로 부상하면서 지금까지 확립된 방사선기술을 바탕으로 생명과학기술, 환경기술, 우주기술, 나노기술 등을 융·복합시킨 새로운 개념의 과학 기술과 신제품을 개발하고자 하는 노력에 가속도가 붙기 시작하였다.

물질의 화학적, 물리적 구조와 특성을 변화시키는 이온화 방사선의 특성을 이용하여 다양한 생물소재로부터 획득한 생물분자의 구조를 변형시킴으로써 기능성 또는 고부가가치 물질을 개발할 수 있다. 방사선 조사를 통해 구조변환된 생물분자가 향상된 항산화능이나 항암능력을 가질 경우 식품, 의료, 산업적 이용가치가 창출되는 것이다. 지금까지 단백질이나 폴리펩티드, 다당류, 이차 대사산물 등을 대상으로 수행된 기초연구의 결과를 바탕으로 새로운 기능성 물질을 개발하고자 하는 노력이 필요한 시점이다.

모든 생명체는 인공 및 자연 방사선과 여러 가지 물리·화학적 요인의 영향을 동시에 받으면서 살고 있다. 다양한 산업 분야에서 이온화 방사선이 가지고 있는 특성을 긍정적 이용하기 위한 노력이 증대되고 있으며 특히 생물·화학적 이용 효율을 증가시켜 특정 목적을 달성하기 위해서 방사선 기술과 여타의 기술을 병용하는 사례가 증가되고 있다. 방사선 기술은 BT, ET, NT 등 주요 기술과 연계성이 클 뿐 만 아니라 상호보완적이기 때문에 독립적인 국가 핵심기술의 하나로 인정되고 있다. 두 가지 이상의 기술을 복합한 방사선 융·복합기술은 방사선을 조사하여 그 영향을 단순히 이용하던 단편적 기술의 한계를 극복하고 새로운 기술로 도약할 수 있는 다제간 신기술로서 다양한 이용범위를 가지며 산업적 파급효과가 높은 동시에 경제적 효과가 큰 강점 기술이다. 하지만 선진국 및 국내 모두 그 기술기반이 취약한 상태이며 학술적 근거도 미비된 상태로 취약한 기술기반을 확립하고 학술적 근거를 강화하기 위한 연구개발 또한 시대적으로 요구되는 과제이다.

지금까지 방사선의 단순 조사 위주로 이용해 오던 평면적 기술에서 탈피하여 방사선 기술을 기반으로 하여 생명과학기술, 환경기술, 정보기술, 문화기술 등을 복합적으로 활용하여 한 차원 높은 방사선 융·복합 기술을 개발할 필요가 있다. 따라서 본 연구는 방사선 융합 기술을 이용하여 생물분자의 과학적, 산업적 이용가치를 창출하는 한편 방사선의 긍정적 이용 효율을 극대화하기 위한 체계 정립에 역점을 두고 있다. 본 연구를 통해 방사선융합기술을 확립하여 방사선 이용의 새로운 장을 마련함으로써 국민의 삶의 질 향상에 기여하는 한편 국가 경쟁력을 높이는 계기를 마련하고자 한다.

KAERI

제 2 장 본 론

제1절 방사선 구조변화 기술이용 실크 피브로인 단백질 개발

1. INTRODUCTION

Silks are generally defined as protein polymers that are spun into fibers by certain *Lepidoptera larvae* such as silkworms, spiders, scorpions, mites, and flies (Kaplan *et al.*, 1998). Silk derived from silkworm *Bombyx mori* is a natural protein that is mainly made of sericin and fibroin proteins (Masahir *et al.*, 2000).

In olden days, fibroin was only used for clothing silk, but it has recently been suggested as a biomaterial and a matrix for biomedical applications. The unique physicochemical properties of fibroin (Minoura *et al.*, 1990) have enabled its various utilizations such as cell culture matrices (Minoura *et al.*, 1995), enzyme-immobilizing membranes (Qian *et al.*, 1997) and an oral dosage gel form (Hanawa *et al.*, 1995). Several biological activities of silk fibroin have also been reported. Lowered blood cholesterol, glucose levels, and alcohol absorption were observed in fibroin-fed rats (Akai, 1999), and sulphated fibroins showed an anti-HIV activity (Goth *et al.*, 2000). In addition, the hydrolysates of silk fibroin have been considered for applications as a functional material for foods, cosmetics, and pharmaceutical preparations (Kim *et al.*, 1996).

Gamma irradiation is a useful technology for an improvement of the storage and hygiene of food and also found to have importance in the medical and beauty care industry (Byun, 1994). Also, there have been several reports for a change of the structure and physiological properties of protein by an irradiation (Lee *et al.*, 2005). Silk fibroin is a useful model to study for the changes of molecular structure and physiological activities by gamma irradiation. However, there are only a few reports on the effect of irradiation on the production of fine powder from silk (Hidefumi *et al.*, 2005; Masuhiro *et al.*, 2003).

Thus, this investigation was conducted to examine the changes in the molecular structure and physiological activities of fibroin by gamma irradiation and was undertaken to explore the possibilities of gamma-irradiated fibroin as a material for functional food stuff and cosmetics.

2. MATERIALS AND METHODS

Preparation of Fibroin

Cocoons were supplied by Nuero Ltd. (Daegu, Republic of Korea). To remove sericin, 50 g of cocoons were cut into small pieces and boiled in 2.5 L of 5% (w/v) Na_2CO_3 for 1 h and filtered through a filter paper. The remaining sericin and Na_2CO_3 were removed by washing the residue with hot water three times. The fibroin was solubilized using a solution of $CaCl_2$ -/H_2O - ethanol (Oguz *et al.*, 2005). A mixture containing 226.4 g $CaCl_2$, 346 g distilled water and 280 ㎖ ethanol was added to 35 g fibroin residue and heated at 100℃ for 2 h. The dialysis (MW cutoff of 1000, Spectrum Laboratories Inc., CA, USA) was carried out five times, and then the fibroin was dried in a vacuum freeze dryer to obtain fibroin powder. The purified fibroin powder was stored in a refrigerator at 4℃ for further experiment.

Gamma irradiation of fibroin

Fibroin was dissolved into a concentration of 5 ㎎/㎖ (w/v) in double distilled water (DDW). Sericin and fibroin solution was irradiated at 0, 5, 10, 50, 100, 150, and 200 kGy, in a cobalt-60 irradiator (IR-79, Nordion International Ltd.) with a 300 kCi source strength and operated at a dose rate of 10 kGy/h. The gamma-irradiated sericin and fibroin solutions were stored in a refrigerator at 4℃ for the subsequent experiment.

Gel permeation chromatography

The distribution of the molecular weight of fibroin was determined by a gel permeation chromatography (GPC). GPC measurements were performed by a Waters GPC system equipped with a Waters 515 pump, 2 PLaqagel OH Mixed (7.8 300 mm) column and a Waters 2410 refractive index detector. The column was operated at 40℃ and eluted with distilled water at a flow rate of 1 ㎖/min. The column was calibrated by a dextran standard at a concentration of 0.1% (w/w).

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

The protein concentration of fibroin was assayed by using the BCA method (Krieg *et al.*, 2005). Electrophoresis was carried out by using precasted 4 ~ 20% Nu-PAGE Bis - Tris gels (Invitrogen, San Diego, CA) at 100 V in a Nu-Page MES SDS running buffer system (Invitrogen Co.) according to the manufacture's instructions. A SeeBlue Plus2 Pre-stained Standard marker was purchased from Invitrogen Co. and was used to determine the molecular masses of the protein bands. The marker consisted of protein band with the following molecular masses: 188, 98, 62, 49, 38, 28, 17, 14, 6, and 3 kDa. The gel was stained for visualization with Commassie Brilliant Blue R-250.

Circular dichroism

Circular dichroism (CD) spectra were obtained using a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) fitted with a 150-W xenon lamp. Far-UV spectra were registered in the range of 160 ~ 280 nm. Sample (0.2 ㎎/㎖) was analyzed in DDW. A sample compartment was purged with nitrogen gas and a 1-mm cuvette was used. Triplicate scans were averaged, and the spectrum for the DDW background was subtracted. CD spectra were represented in terms of a mean residue ellipticity (degree cm^2/dmol).

UV spectrum

Gamma-irradiated sericin and fibroin solution were scanned by using a spectrophotometer (UV-1601PC, Shimadzu, Tokyo, Japan). The UV spectra were recorded between 200 and 800 nm. A total of 3 ㎖ of a sample (0.1 ㎎/㎖) was assayed on a UV-VIS spectrophotometer with a quartz cell.

Physiological activities of silk sericin and fibroin

Measurement of the DPPH radical scavenging activity of gamma irradiated silk fibroin

The free-radical scavenging activity using the 2.2-diphenyl-1-picryl-hydrazil (DPPH) reagent was measured using the method described by Amarowicz *et al.* (2004), with a slight modification. To 0.5 ㎖ of fibroin dissolved in DDW at a concentration of 0.2 ㎎ fibroin /㎖, the same volume of freshly prepared DPPH radical in methanol (0.1 mmol) was added and vortexed. After a reaction for 25 min at room temperature (25℃) in the dark, the reaction mixtures were centrifuged at 6400 rpm for 5 min. The decolorization of the supernatant was assayed at 517 nm (A_{517}) by a spectrophotometer (UV-1601PC, Shimadzu) and was compared with a blank control containing a fibroin solution and pure methanol instead of DPPH. A blind control containing DPPH and distilled water instead of a fibroin solution was also assayed. Equation (1) was used for the calculation of the free-radical scavenging activity;

$$\text{Scavenging activity (\%)} = (1 - A^{sample}_{517}/A^{blind}_{517}) \times 100 \quad (1)$$

where the scavenging activity refers to the free-radical scavenging percentages, A^{sample}_{517} refers to the absorbance of a sample, and A^{blind}_{517} refers to the absorbance of the blind control.

Measurement of tyrosinase inhibition by gamma-irradiated silk fibroin

Samples of gamma-irradiated sericin and fibroin solution (0.2 ㎖) were added to a reaction mixture containing a 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma Chemical Co., St.Louis, Mo, USA) solution, a 67 mM sodium phosphate buffer (pH 6.8), and mushroom tyrosinase (final concentration of 100 unit/㎖, Sigma Chemical Co.). The reaction mixture was incubated at 25℃ for 15 min. The amount of dopachrome produced in the reaction mixture was determined at 475 nm (A_{475}) by a spectrophotometer (UV-1601PC, Shimadzu). Equation (2) was used for the calculation of inhibitory effect on the tyrosinase (%).

$$\text{Inhibition (\%)} = (1 - A^{sample}_{475}/A^{control}_{475}) \times 100 \quad (2)$$

where $A^{control}_{475}$ refers to the absorbance with DDW instead of the fibroin solution.

Determination of melanogenesis in B16BL6 cells

In the current study, melanin content was used as an index of melanogenesis. Estimations of melanin content were performed using a modified method of Bilodeau *et al.* (2001). In short, B16 cells (1×10^5) were plated on 24-well dishes and incubated in the presence of 100 nM α-MSH. Cells were then incubated for 72 h with non-irradiated sericin/fibroin and gamma-irradiated fibroin at concentrations of 62.5 ㎍/㎖. After washing twice with PBS, samples were dissolved in 100 ul of 1 N NaOH and water-bath at 70℃. The Absorbance at 405 nm was compared with a standard curve for synthetic melanin.

Immune activities of silk sericin and fibroin

Cell culture

The test cell line of B16BL6 (skin cancer), AGS (stomach cancer), HT-29 (colon cancer) and RAW264.7 (macrophage cell) were purchased from the Korean Cell Line Bank (KCLB). B16BL6 and RAW264.7 were adapted in EMEM and DMEM medium containing 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin. AGS and HT-29 were adapted in a RPMI-1640 medium containing 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin and then cultured at 37℃ in 5% CO_2.

MTT assay

Cytotoxicity of the cancer cells by sericin and fibroin were detected by the MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide, sigma] assay method. Each cancer cell was inoculated into a 96 well plate with 90 ㎕ of 3×10^4/well medium and 10 ㎕ of fibroin. After 24 h, 30 ㎕ of 5 ㎎/㎖ MTT in PBS was added to each well and the plate was incubated at 37℃ for 2 hrs. The plate was then centrifuged and the medium was removed. 100 ㎕ of dimehylsulfoxide (DMSO,

sigma) was then added. After incubation at 37℃ for 5min, the absorbance of the sericin and fibroin-treated cells was mesured at 540 nm by using microplate reader.

Experimental animals

C57BL6 and BALB/c mice (7 weeks) were purchased from Orient Inc. (Charles River Technology, Seoul, Korea). The mice were housed in polycarbonated cage and were fed with a standard animal diet and water *ad libtum*. Research was conducted according to principles enunciated in the 'Animal Care Act', prepared by the Ministery of Agriculture and Forestry, Korea.

Macrophage cell culture

Peritoneal macrophages were harvested from thioglycollate-treated mice as described previously (Kraatz *et al.*, 1999). Cells (1×10^6/㎖/well) suspended in complete RPMI-1640 medium were plated into 48-well culture plates. After 2 h incubation, non-adherent cells were removed by washing with PBS, the adherent macrophages were co-incubated with the various concentration of fibroin for 24 h.

Assessment of NO Production

NO levels were determined using the Griess Reagent, a common experimental measure for assessing nitrite, a metabolic byproduct of nitric oxide. Equal amounts of supernatant and Griess Reagent (100 ㎕) were mixed according to manufacturer's instructions. The absorbance was measured at 595 nm using an ELx808 absorbance microplate reader (Bio Tek, Winooski, VT).

Experimental tumor induction and gamma-irradiated fibroin treatment

Tumors were induced by using B16BL6 cells. The B16BL6 cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 at a 75 cm^2 cell culture flasks (Corning, New York) in EMEM culture medium, supplemented with 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin. Experimental tumor induction

of B16BL6 melanoma cells (4×10^4) was assessed by *i.d* injection of tumor cells into C57BL6 mice (4 groups, 5 mice per a group). In the tumor growth experiment, mice were given oral administration (2.5 ㎎/head) of non-irradiated fibroin and gamma-irradiated fibroin (50, 150 kGy)for 7 days. The mice were sacrificed as 14 days after tumor inoculation.

Isolation of Splenocytes from Animals

Spleen cells were obtained by gentle disruption of the spleen (C57BL6 & BALB/c mice) and repeated pipetting was done to attain single cell suspension. After the cells were twice washed in ice cold RPMI-1640 medium developed by Moore *et al.* (1985) at Roswell Park Memorial Institute, lymphocytes were separated from erythrocytes using ice cold distilled water for 30 s and then counted for cell viability (90%). Splenocytes were maintained in RPMI-1640 medium with 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin at 37°C under 5% CO_2 in air (Cederbrant *et al*., 2003).

Spleen cell culture

Spleen cell suspensions were plated in 96-well tissue culture plates at a final concentration of 1×10^6 cells/well maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin and then cultured at 37℃ in 5% CO_2. Cultured supernatant harvested after 24 h and stored at -70℃ for cytokine evaluation

Cell Proliferation Assay

Spleen cell proliferation was detected by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay method. Following an incubation of the cells with fibroin for 24 h, 30 ㎕ of 5 ㎎/㎖ MTT in PBS was added to each well and the plate was incubated at 37℃ for 2 h. The plate was then centrifuged and the medium was removed. Then formazan crystals were dissolved using 100 ㎕ of dimethylsulfoxide (Sigma, St. Louis. Mo). After incubation at 37℃

for 5 min, the optical density was measured at 595 nm in automated micro plate ELISA reader.

Cytokines Assay

Supernatants cultured for 24 h were evaluated by BD OptEIA™ cytokine detection kit by following the manufacturer's instruction (BD Biosciences, San Jose, CA). Cytokines Sandwich enzyme-linked immunosorbent assay (ELISA) for IL-6, IL-2, IL-12, IFN-γ and TNF-α was carried out in triplicate using 96-well ELISA plates (costar 3595, Corning, NY)

Natural killer cell and $CD4^+/8^+$ T lymphocytes population

Spleen cells were obtained by gentle disruption of the spleen and the population of NK cells from BALB/c evaluated by FACS (Fluorescence activated cell sorter). The population of $CD4^+$ and $CD8^+$ T lymphocytes from BALB/c was analyzed by FACS. FACS-contents: Phycoerythrin-Cy7 (PE-Cy7) anti-mouse pan-NK cells, Fluorescein isothiocyanate (FITC) anti-mouse $CD4^+$ (L3T4), Phycoerythrin (PE) anti-mouse $CD8^+$ (Ly-2).

Statistical analysis

The data were analyzed by the Statistical Package for the Social Science (SPSS Inc. Chicago, IL) program. Differences among the mean values were obtained by Duncan multiple comparison tests and LSD at $p < 0.05$.

3. RESULTS AND DISCUSSION

Effect of gamma irradiation on molecular weight distribution of fibroin

The effect of irradiation on molecular weight distribution of the gamma-irradiated fibroin was investigated by using a gel permeation chromatography (GPC) and a sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

Fig. 1 represents the average molecular weight changes of fibroin as an expression of the radiation effect under different doses. The average molecular weight of the untreated fibroin was found to be 5 kDa. The average molecular weights of the fibroin gamma-irradiated at the doses of 5 and 10 kGy were approximately 320 and 576 kDa. The molecular weight observed in fibroin gamma-irradiated with 50, 100, 150, and 200 kGy could not be calculated because of the upper limit of GPC. The molecular weights of fibroin gamma-irradiated at 50 to 200 kGy were higher than the GPC calibration range. In these results, the molecular weight of the gamma-irradiated fibroin was increased as the irradiation dose was increased. Many previous reports depicted that there was a phenomenon of a cross-linking and on ahydrophobic interaction between proteins, due to an irradiation (Garrison, 1987; Kume & Matsuda, 1995; Puchala & Schessler, 2004). Therefore, our data indicated that fibroin might be fairly aggregated into the high-molecular-weight fragments during irradiation.

SDS-PAGE results suggested that the fibroin represented a family of proteins with diverse molecular weight distributions. Electrophoretic pattern of the gamma-irradiated fibroin from the doses of 5 to 200 kGy are shown in Fig. 2. SDS-PAGE analysis of the non-irradiated fibroin showed a broad smear at a position around 5 to 50 kDa. Gamma-irradiated silk fibroin at 5 kGy showed higher molecular weight (38 ~ 188 kDa) than that of 0 kGy and gamma-irradiated (10 kGy) silk fibroin showed broad smear at a position around 90 to more 188 kDa. Gamma-irradiated fibroin at 5 to 10 kGy showed a higher molecular weight distribution than that of 0 kGy, but the molecular weight observed in fibroin gamma-irradiated with 50, 100, 150, and 200 kGy were not observed any standard marker range except for loading point (starting point). In other words, gamma-irradiated fibroin at the doses ranging from 50 to 200 kGy showed that the molecular weight was more 188 kDa. From these results, as the radiation dose was increased, the proteins bands aggregated to higher molecular weight and the protein bands at lower molecular weight disappeared. These results also indicate that the gamma-irradiated fibroin revealed the phenomena of aggregation and hydrophobic interaction on the SDS-PAGE, similar to the GPC data shown in Fig. 1.

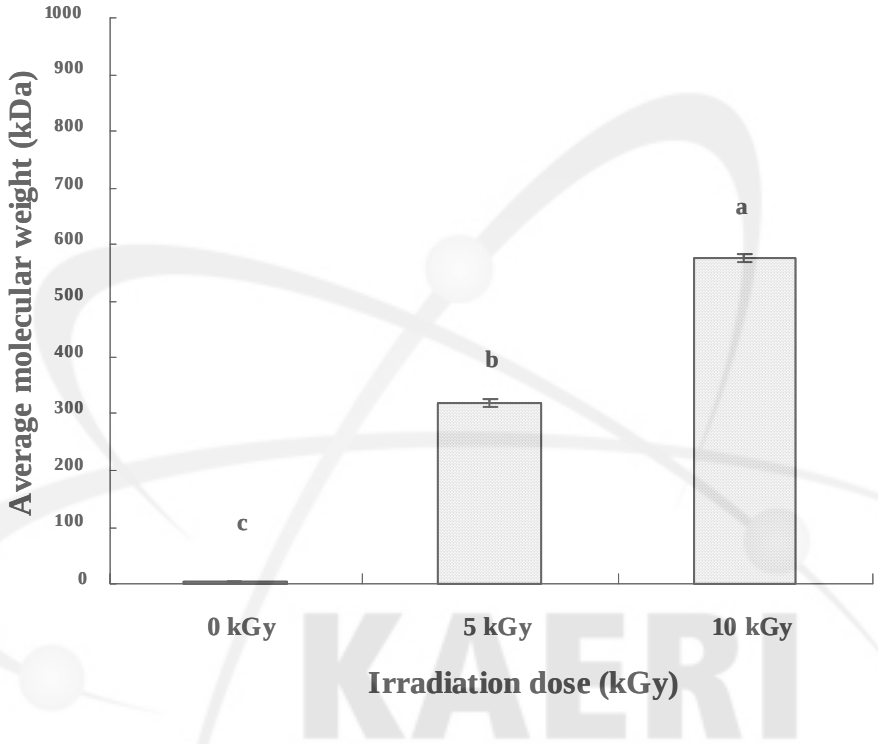

Fig. 1. GPC analysis for the average molecular weight distribution of the gamma-irradiated fibroin at different doses. Bars represent the mean ± S.D. The letters mean the statistically significant difference compared with the control ($p < 0.05$).

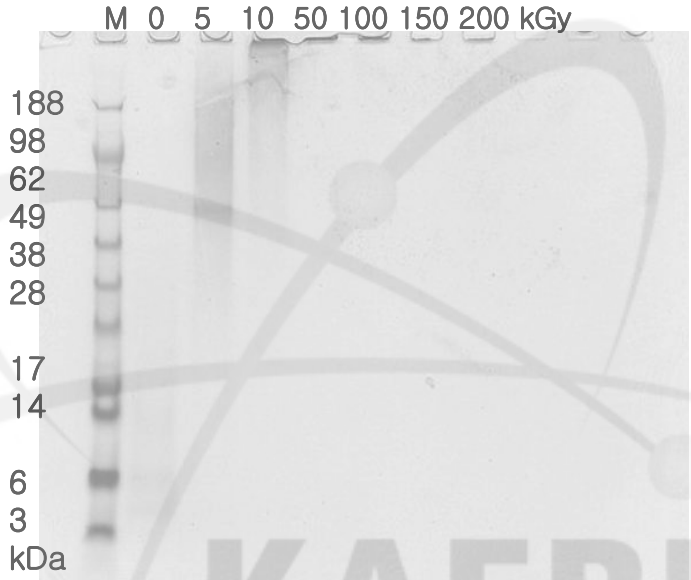

Fig. 2. SDS-PAGE of gamma-irradiated fibroin at 0, 5, 10, 50, 100, 150 and 200 kGy, respectively. M indicates standard markers and the numbers on the left side indicate the molecular weight (kDa) of the standard marker.

Analysis of secondary structure of gamma-irradiated fibroin by circular dichroism

Circular dichroism (CD) spectroscopy measures the differences in the absorption of a left-handed polarized light versus a right-handed polarized light, which arises due to structural asymmetry. CD spectroscopy can be used to determine a protein's secondary structure in far-UV spectral region (190 ~ 250 nm). At these wavelengths, a chromophore is a peptide bond, and signal arises when it is located in a regular, folded environment, α-helix, β-sheet, and random coil structures that provide the characteristic shape and magnitude of CD spectrum. It is commonly known that two negative peaks at 208 and 220 nm are the characteristics of α-helix secondary conformation of proteins and that at 214 nm are the characteristics of a β-sheet secondary conformation of proteins (Chang *et al.*, 1978; Mayer, 1998).

Fig. 3 shows the change in the fibroin's secondary structure caused by irradiation. When the radiation dose was increased, the content of α-helix secondary conformation increased at 5 to 10 kGy and then decreased at 50 to 200 kGy. On the other hand, the contents of β-sheet, β-turn, and random coil increased with an alteration in α-helix secondary conformation. The β-turn conformation comprised four residues and forms a hydrogen bond between the carbonyl group of the first residue and the NH group of the fourth residue. This conformation is frequently found in a globular protein surface, a common structural element in globular proteins that results in the folding due to the reversal in the direction of a polypeptide chain (Shin *et al.*, 1997). Thus, the β-turn can act as one of the key elements indicating the folded structures of native proteins (Chang *et al.*, 2002). The appearance of a change in the β-turn conformation for fibroin treated with an irradiation implies that the change was probably associated with a molecular aggregation of the protein induced by irradiation (Lee *et al.*, 2003).

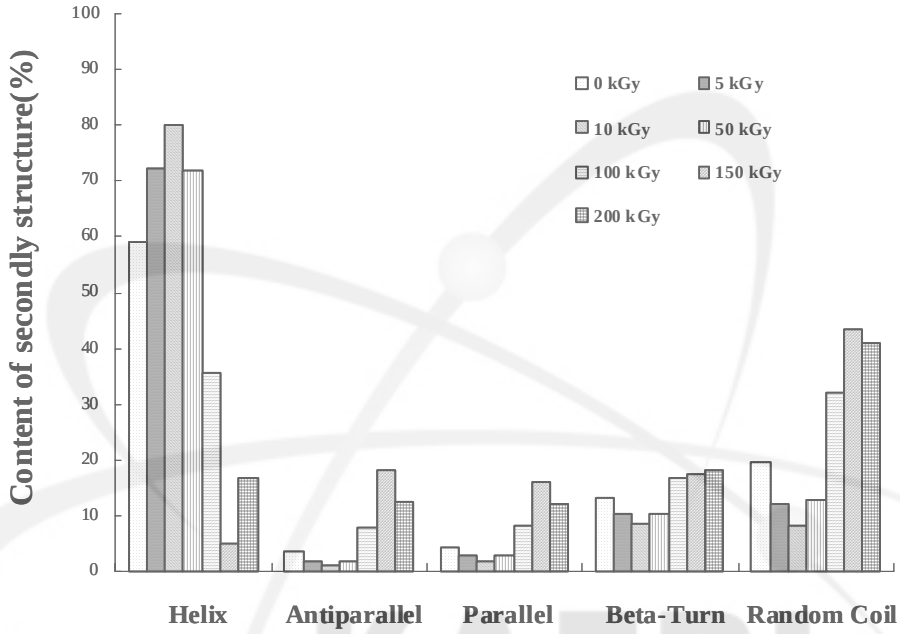

Fig. 3. Analysis of the secondary structural conformations by the far-UV CD spectra of the gamma-irradiated fibroin at 0, 5, 10, 50, 100, 150 and 200 kGy, respectively.

Changes in UV spectrum of fibroin by gamma irradiation

UV absorption pattern was investigated to confirm changes of conformation of gamma-irradiated fibroin with 5 to 200 kGy compared with unirradiated fibroin. UV absorption spectra revealed a conformational change due to the absorbance by side chains of aromatic amino acids on the protein's surface. The three amino acids, such as phenylalanine, tyrosine, and tryptophan, carry aromatic side chains. These aromatic amino acids, like most compounds carrying conjugate rings, strongly absorb light in the near-ultraviolet region of a spectrum (Wetlaufer, 1962). Tyrosine and tryptophan account for most of the UV absorbance by proteins in the region around 280 nm. Phenylalanine mostly reveals the absorption at 260 nm. This characteristic is frequently used for the analytical detection of proteins (Mathews *et al*., 1999).

As shown in Table. 1, the absorbance of the gamma-irradiated fibroin increased at 260 and 280 nm as the radiation dose was increased from 0 to 10 kGy, but the absorbance of the gamma-irradiated fibroin between 50 and 200 kGy has approximately similar results around 1.802 at 280 nm. These results indicated that the absorbed light in the ultraviolet region of the spectrum of amino acid such as tyrosinase and tryptophan is similar and these changes in the UV absorption reveal a conformational change caused by an irradiation, suggesting that the internal aromatic amino acids of a protein were exposed due to a disruption in the conformation process and that the extent of the disruption depended on the irradiation dose.

Table 1. UV absorption spectra of the gamma-irradiated fibroin at 0, 10, 50, 100, 150 and 200 kGy, respectively

Absorbance	Dose (kGy)						
	0	5	10	50	100	150	200
OD (260 nm)	1.632	1.764	1.804	1.855	1.855	1.851	1.852
OD (280 nm)	1.766	1.796	1.802	1.807	1.812	1.803	1.802

DPPH radical scavenging capacity of gamma-irradiated fibroin

2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical with a characteristic absorption at 517 nm, was used to investigate the radical scavenging effects of fibroin. The DPPH radical scavenging capacity of the gamma-irradiated fibroin is shown in Fig. 4. The DPPH radical scavenging capacity of the gamma-irradiated fibroin at 5 kGy was found to be higher than that of the non-irradiated fibroin and all of the gamma-irradiated fibroins between 10 to 200 kGy showed a lower radical scavenging capacity compared with that at 5 kGy. The results suggest that the maximum dose for an antioxidant effect of the gamma-irradiated fibroin is 5 kGy.

In a previous study, Choi *et al.* (2000) provided evidence on the antioxidant activity of fibroin by *in vivo* experiment. These results indicated that gamma irradiation increased the DPPH radical scavenging capacity of fibroin by protein aggregation and by the structural changes that are produced. Iskender & Ahmet (2007) reported a change in the antioxidant activity of a protein extract due to a structural change and studies on the mechanism of the antioxidant action of fibroin are in progress.

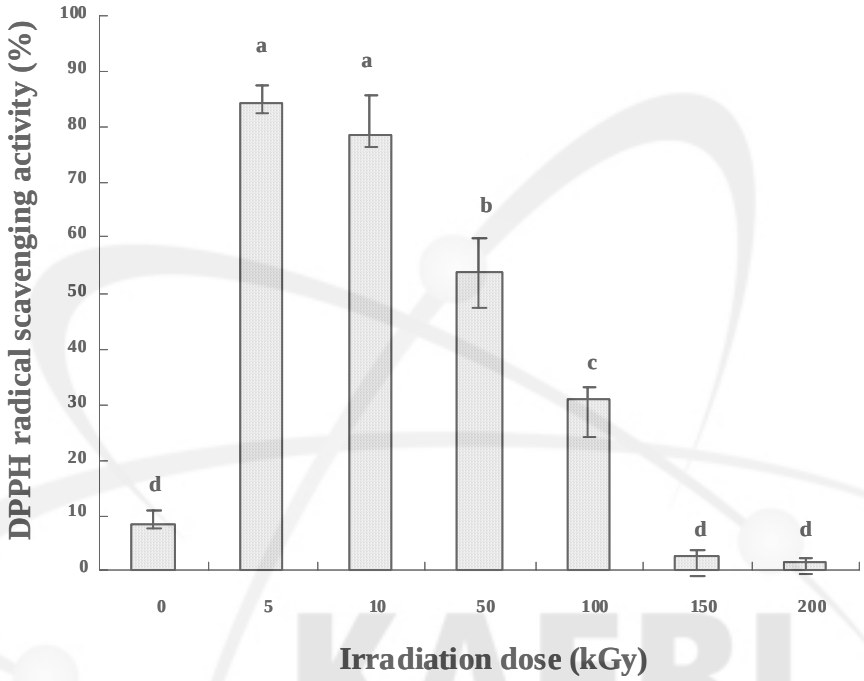

Fig. 4. DPPH radical scavenging effect of the gamma-irradiated fibroin at 0, 5, 10, 50, 100, 150 and 200 kGy, respectively. Bars represent the mean ± S.D. The letters indicate the statistically significant difference compared with the control ($p < 0.05$).

Tyrosinase inhibitory effect of gamma-irradiated fibroin

Tyrosinases are widely distributed in nature; they are found both in prokaryotic as well as in eukaryotic microbes, in mammals and plants (Emilia *et al.*, 2007). The melanin pigment in human skin exhibits a major defense mechanism against damage caused by UV light, but abnormal pigmentation such as melasma, freckles, senile lentigines and other forms of a hyper-pigmentation are often considered undesirable (No *et al.*, 1999). Tyrosinase is responsible for the biosynthesis of pigment melanin in human skin, and tyrosinase inhibitors have important roles in the cosmetics industry due to its skin-whitening effects (Dooley, 1997; Huang *et al.*, 2005). The inhibitory effect of a gamma-irradiated fibroin on tyrosinase was shown in Fig. 5. Although non-irradiated fibroin showed a tyrosinase inhibition effect, the gamma-irradiated fibroin showed a stronger inhibitory effect on the activity of tyrosinase than the non-irradiated fibroin. The inhibitory effect of tyrosinase also increased depending on the irradiation dose. In a previous study, fibroin treated by acid or enzymatic hydrolysis is applied as functional food, cosmetic, medical supplies (Kim *et al.*, 1996), but the major cause for the increase in the inhibitory effect on tyrosinase due to gamma irradiation is elusive at present and is being studied.

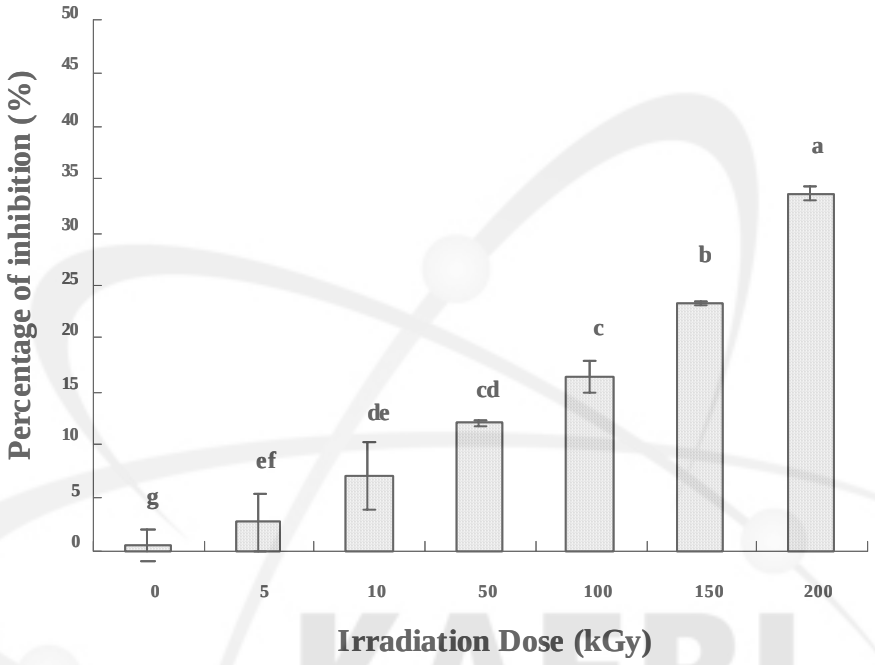

Fig. 5. Effect of gamma-irradiated fibroin on tyrosinase inhibition at 0, 5, 10, 50, 100, 150 and 200 kGy, respectively. Bars represent the mean ± S.D. The letters mean the statistically significant difference compared with the control ($p < 0.05$).

Inhibitory effect of melanogenesis by gamma-irradiated fibroin in B16BL6 cells

Skin pigmentation contributes significantly to the health and well-being of an individual. Pigment synthesized by cutaneous melanocytes protects the individual from various environmental assaults and potential cellular injury that can cause cancer and aging of the skin (Sturm, 2002). Melanogenesis is a multistage process involving melanin synthesis, melanin transport, and melanosome release (Noriko *et al.*, 2007). The melanin pigment in human skin exhibits a major defense mechanism against damage caused by UV light, but abnormal pigmentation such as melasma, freckles, senile lentigines, and other forms of a hyper-pigmentation are often considered undesirable (No *et al.*, 1999). This study is to supply the inhibitory effect of tyrosinase by gamma-irradiated fibroin. Fig. 6 showed the results of the inhibitory effect of melanogenesis by gamma-irradiated fibroin. Dose-dependent inhibitory effect of the irradiated fibroin in cultured B16BL6 melanoma cells was observed as compared with non-irradiated fibroin and was also observed that gamma-irradiated fibroin (100 kGy) has stronger inhibitory effect than Kojic acid, positive control.

In the development of new skin care drugs, considerable effort has been expended in the search for natural substances and their use in the development of skin care cosmetics has recently been emphasized. From these results, it could be concluded that the gamma-irradiated fibroin might be a potential candidate as a value-added product in cosmetic industry.

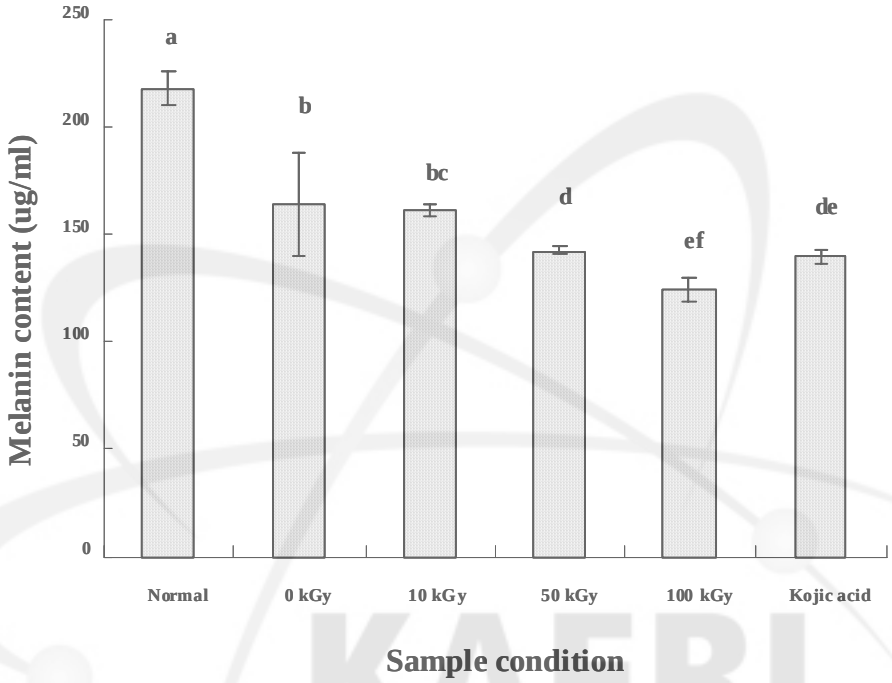

Fig. 6. Inhibitory effect of melanogenesis by gamma-irradiated fibroin at 0, 10, 50 and 100 kGy on B16BL6. Sample final concentration is 62.5 ㎍/㎖. Normal include D.W without fibroin solution. Bars represent the mean ± S.D. The letters mean the statistically significant difference compared with the control ($p < 0.05$).

Cytotoxicity of gamma-irradiated fibroin in cancer and immune cells

In the present investigation HT-29 (colon cancer), B16BL6 (melanoma) and AGS (stomach cancer) were used to determine the cytotoxicity of gamma-irradiated fibroin. Inhibitory effects of cell survival in cancer cell by gamma-irradiated fibroin with 5 to 200 kGy are shown in Fig. 7. To know this inhibitory effect of gamma-irradiated fibroin on cancer cell survival the MTT reduction assay method was used. As reported, Lixia *et al.* (2007) reported that protein of silk or housefly has got anti-tumor activity and Cheema *et al.* (2007) reported that silk fibroin was more efficacious in suppressing the growth of Her2/nue over-expressing brest cancer cells MDA-MB-453. From this investigation, cytotoxicity of gamma-irradiated fibroin was increased with increase of irradiation dose. The cytotoxic effect of gamma ray exposed fibroin on HT-29, B16BL6 and AGS were 30%, 40% and 83% respectively compared with 0 kGy. The 150 kGy fibroin was found to have significant cytotoxic effect.

These results indicated that as radiation dose increases, cancer cell inhibitory effect is also increased. Further investigation was done to determine its effect on RAW 264.7 and spleen cells. Anna *et al.* (2003) reported that silk fibroin protect the human cells from carcinogens. Yang *et al.* (2007) reported that Schwann cells cultured in the silk fibroin extract fluid showed no significant difference in their morphology and cell viability and proliferation increased. The survival of immune cells treated with gamma-irradiated fibroin (5 to 200 kGy) shown in Fig. 8. This investigation showed that as radiation dose increased the immune activities of RAW 264.7 and spleen cell increased irradiation dose dependent manner. These results indicate that cancer cells might be damaged by gamma-irradiated fibroin, on the other hand, in the case of immune cells, there are no damage. From this study, gamma-irradiated fibroin was suitable material to determine the inhibitory effect of tumor growth.

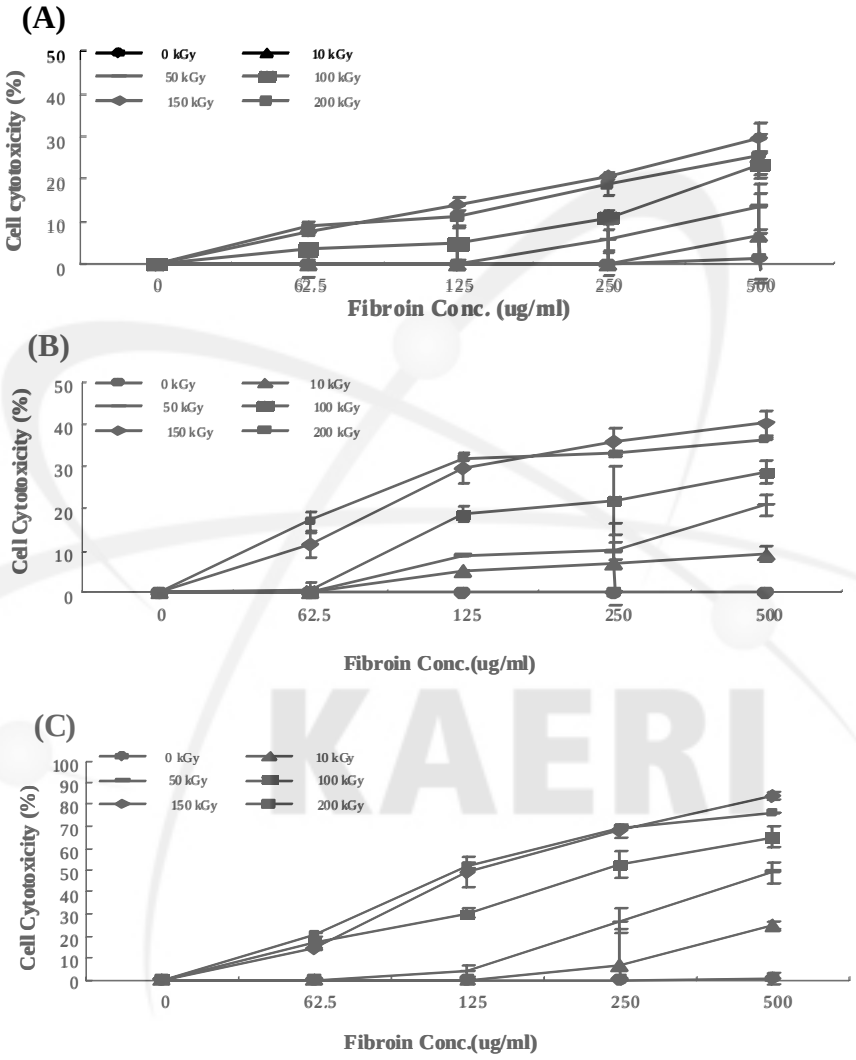

Fig. 7. Cell survival rate of gamma-irradiated fibroin at 0, 10, 50, 100, 150 and 200 kGy on cancer cell. Sample concentration is 500 ㎍/㎖ in DW. (A), HT- 29 (colon cancer); (B), B16BL6 (melanoma); (C), AGS (stomach cancer). Bars represent the mean ± S.D.

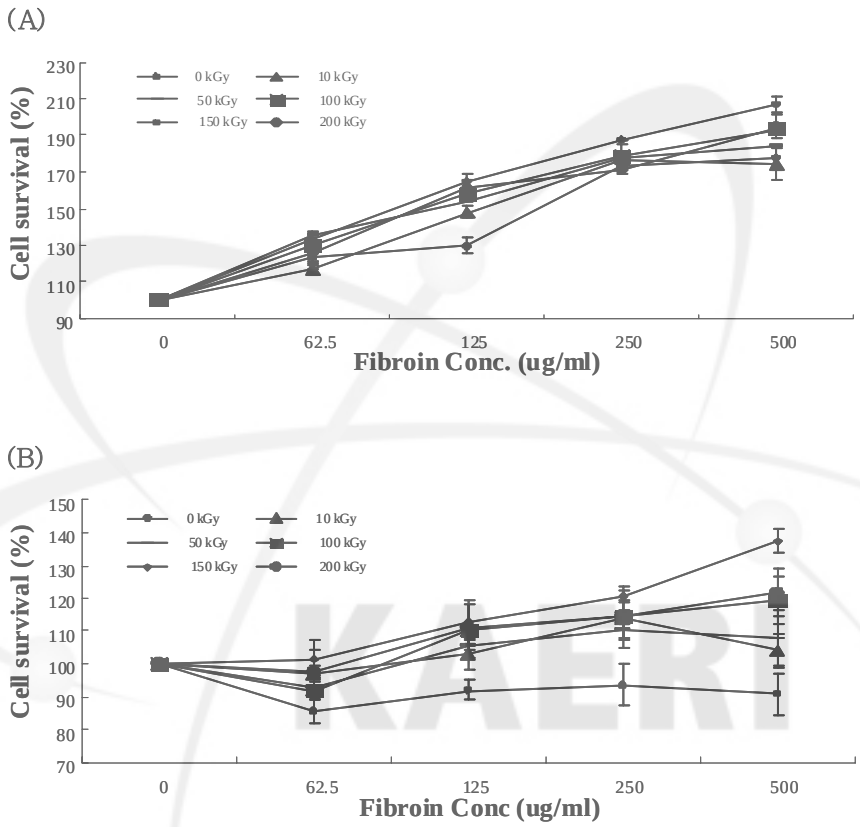

Fig. 8. Cell survival rate of gamma-irradiated fibroin at 0, 10, 50, 100, 150 and 200 kGy on spleen cell (A) and RAW 264.7 (B). Sample concentration is 500 ㎍/㎖ in DW. Bars represent the mean ± S.D.

Immune Response of murine peritoneal macrophage

Macrophages are part of the innate immune system and play important roles in all aspects of immunity. They are exceptionally heterogeneous population of cells. Similar to $CD4^+$ T cells, macrophages can contribute to tumor destruction or to facilitate tumor growth and metastasis, depending on their phenotype. Macrophages that are 'classically activated' by IFN-ɣ and bacterial lipopolysaccharides destroy tumor cells through their production of nitric oxide and Type 1 cytokines and chemokines (Suzanne, 2007).

In the present investigation effects of gamma-irradiated fibroin (50 and 150 kGy) on the production of TNF-α, IL-6 and IL-12 from mouse peritoneal macrophage is shown in Fig. 9. Cytokine results of mouse macrophage response indicated that as radiation dose increases, the effect of immune response is increased. These results indicated that the ability of gamma-irradiated fibroin to induce various cytokines from macrophage may underlie its enhancement of natural immunity and inhibition of tumor growth. In the result of Fig. 10, the production of nitric oxide (NO) from peritoneal macrophage was also increased than that of non-irradiated fibroin. And in the previous report, it is well known that the effecter functions of macrophage NO production includes host-benefit properties such as antimicrobial and tumoricidal activities (Stuehr & Nathan, 1989). Activated macrophages and NK mediate natural immunity against tumors (Barlozzari *et al.*, 1985; Yoon *et al.*, 2004) and gamma-irradiated fibroin induced the secretion of TNF-α, IL-6 and IL-12 from macrophages. Inflammatory cytokines such as IL-12 and TNF-α secreted from macrophages play a role in activating T cells and rejecting tumor cells (Tanigawa *et al.*, 2000; Baxevanis *et al.*, 2000). IL-6, 12 and TNF-α is important in immunoregulation and anti-tumor mechanisms, and has been extensively tested *in vitro* and *in vivo*, as well as in clinical trials for immunotherapy of malignant diseases. IL-12 is produced from phagocytic antigen-presenting cells such as macrophages and dendritic cells in early stages of the immune response. IL-12, a multifunctional cytokine, is critical for eliciting tumor immunity (Hunter *et al.*, 1995; Lee *et al.*, 2003). Therefore, the 150 kGy fibroin was found to significantly inhibit tumor growth. In addition the effect of inhibition on tumor growth and stimulation of cytokine release by gamma-irradiated fibroin could be due to macrophage-mediated cytotoxicity, which may explain its anti-tumor activity.

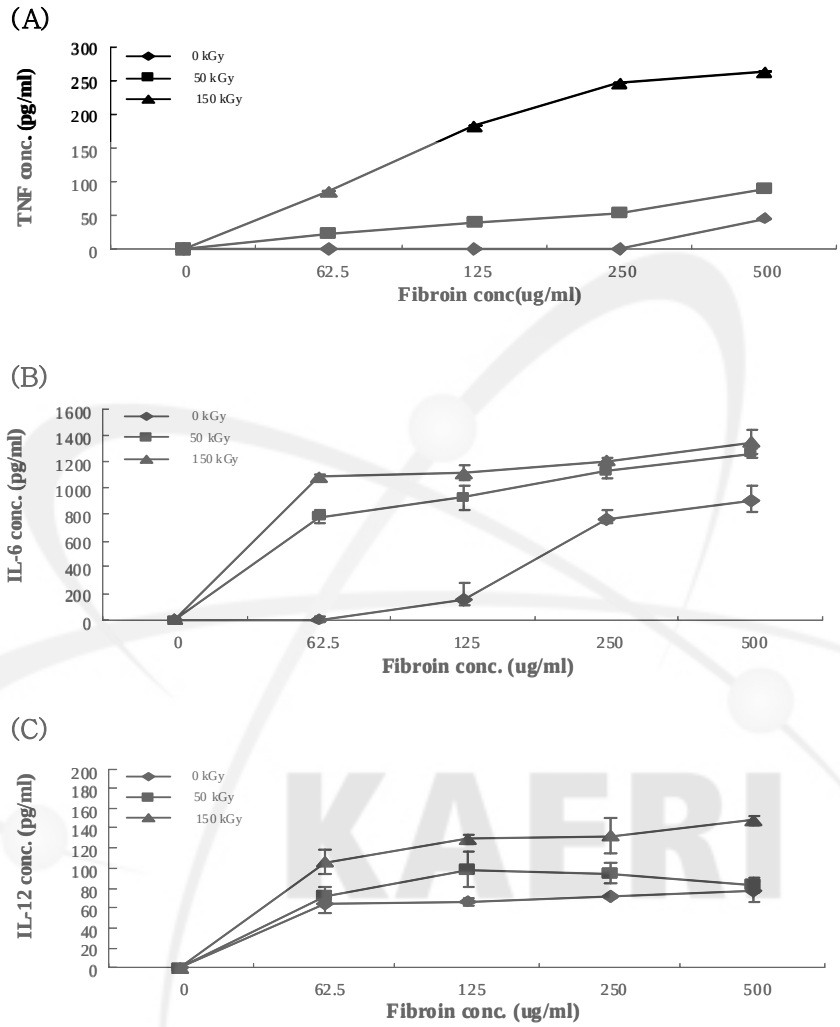

Fig. 9. Effect of non-irradiated and gamma-irradiated fibroin (50 and 150 kGy) on the production of TNF-α (A), IL-6 (B) and IL-12 (C) from murine peritoneal macrophage. Bars represent the mean ± S.D.

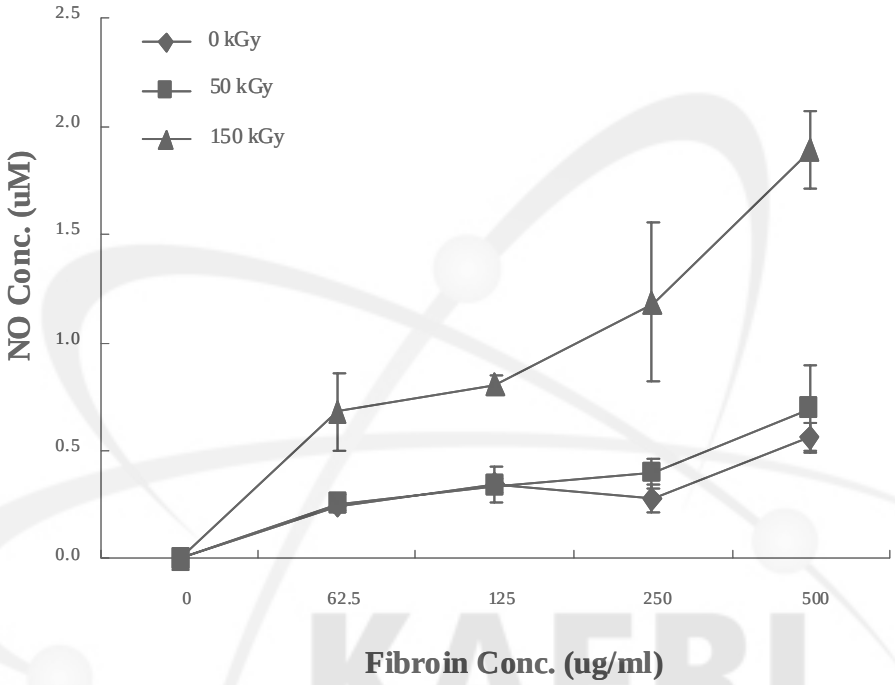

Fig. 10. Effect of non-irradiated and gamma-irradiated fibroin (50 and 150 kGy) on the production of NO from peritoneal macrophage. Bars represent the mean ± S.D.

Effect of gamma-irradiated fibroin on inhibition of tumor growth (*in vivo* response)

Tumor cells are characterized by changes in the profile of protein expression. Such changes may lead to the imbalanced production of proteins relating to the malignancy of the cells, or to the production of mutated proteins with abnormal functions. The altered protein expression in tumor cells may contribute to the cell survival, unregulated proliferation and metastatic nature of the cells (Takahashi *et al.*, 2006).

The immune system has the capacity either to block tumor development and deter established tumors, or to promote carcinogenesis, tumor progression, and metastasis. Which of these conditions prevails depends on the balance between the pro-tumor and anti-tumor mediators of both innate and adaptive immunity (Suzanne, 2007).

In the present investigation the effect of oral administration of gamma-irradiated fibrion (5 and 150 kGy) on the inhibition of tumor (B16BL6) growth in C57BL6 mouse is shown in Fig. 11. And the inhibitory effect of tumor growth by gamma-irradiated fibroin was increased with increase of irradiation dose. The tumor mass of 0 kGy, 50 kGy and 150 kGy fibroin treated mice were 0.28 g, 0.15 g, 0.13 g and 0.06 g respectively. The 150 kGy fibroin was found to have significant inhibition on tumor growth than any other irradiation doses used in the study.

From these results, this study suppose that gamma-irradiated fibroin enhance inhibitory effect of tumor growth owing to innate and adaptive immune system, in which cancer cell-specific and non-specific immunological mechanisms are involved.

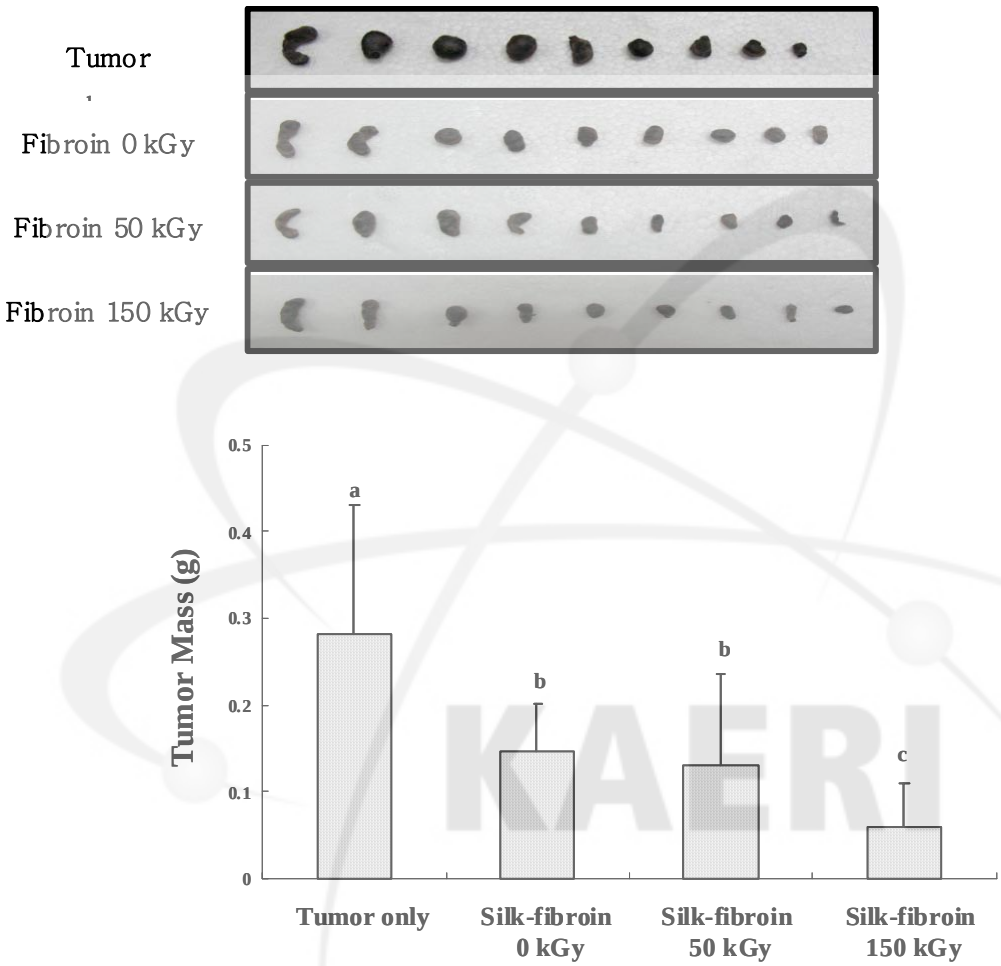

Fig. 11. Effect of oral administration of gamma-irradiated fibrion on the inhibition of tumor (B16BL6) growth in C57BL6 mouse. Results are expressed as the mean ± S.D. The letters indicate the statistically significant difference compared with the control ($p < 0.05$).

Immune Response of murine splenocyte

T-cell from mouse spleen is part of the adaptive immune system and play important role in all aspects of immunity. INF-ɣ and IL-2 secreted from mouse spleen T-cell can stimulate another immune system. Presumably, there are unifying mechanisms that orchestrate immunity towards tumor promotion versus tumor destruction. Since many of the tumor-promoting elements of the immune system are induced by, or themselves cause, inflammation, chronic inflammation may be a key process that polarizes immunity toward a tumor-promoting phenotype (Suzanne, 2007).

In this investigation immune response of cytokine, INF-ɣ and IL-2 and proliferation of spleen cell was assessed in order to determine tumor inhibitory effect of immune system. Proliferation effects of gamma-irradiated fibroin on tumor bearing mouse spleen cell is shown in Fig. 12. The results showed that, as radiation dose increased, the survival rate (proliferation) of spleen cell was increased (irradiation dose dependent) and the group of tumor only showed cell proliferation less than the normal group. Fig. 13 shows the effects of non-irradiated and gamma-irradiated fibroin on mouse spleen cell (IL-2 and IFN-ɣ production). The results indicates that cytokine (INF-ɣ and IL-2) results of T-cells produced from mouse spleen indicated that as radiation dose increases, the effect of immune response also increase.

From these results, this study supposes that gamma-irradiated fibroin enhance inhibitory effect of tumor growth owing to innate immune system of macrophage and adaptive immune system of T-cell.

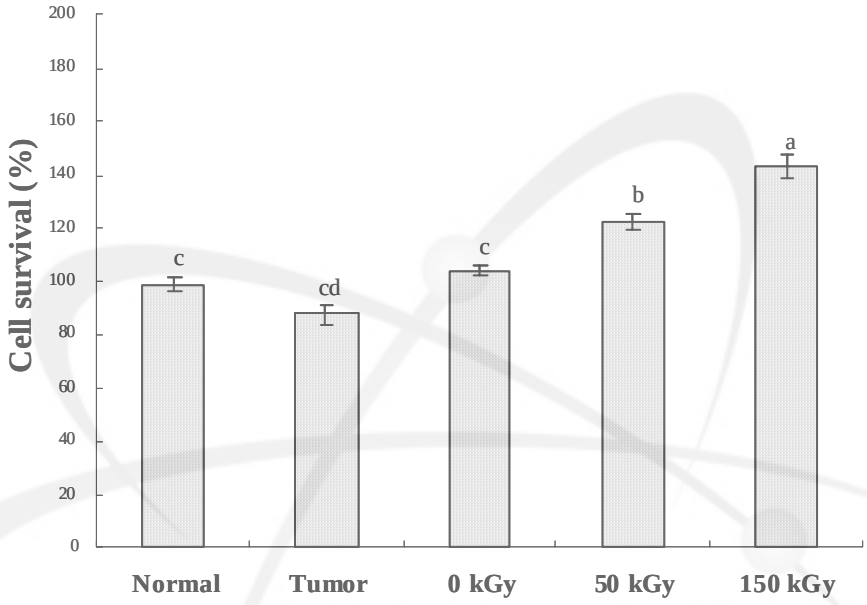

Fig. 12. Effect of non-irradiated and gamma-irradiated fibroin on tumor bearing mouse spleen cell. Sample concentration is 500 ㎍/㎖. Results are expressed as the mean ± S.D. Each experiment was run in triplicate. The letters indicate the statistically significant difference compared with the control ($p < 0.05$).

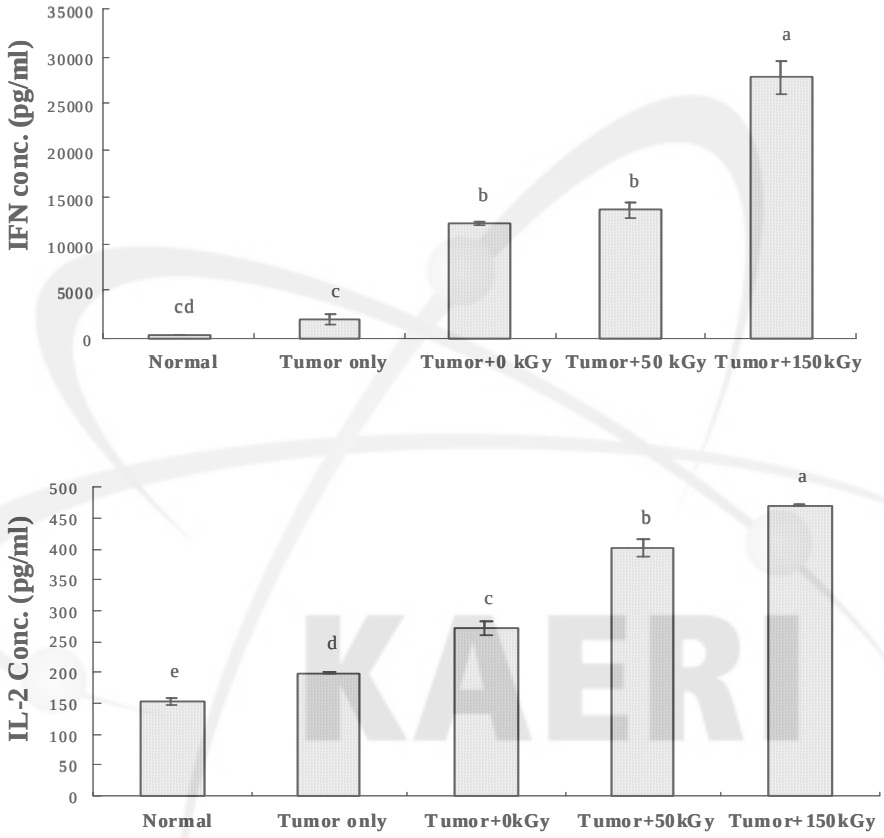

Fig. 13. Effect of non-irradiated and gamma-irradiated fibroin on mouse spleen cell IL-2 and IFN-γ production. Results are expressed as the mean ± S.D. Each experiment was run in triplicate. The letters indicate the statistically significant difference compared with the control ($p < 0.05$).

Immune Response of natural killer cell and $CD4^+$ and $CD8^+$ T lymphocyte population

Natural killer cells (or NK cells) are a type of cytotoxic lymphocyte that constitute a major component of the innate immune system. NK cells play a major role in the rejection of tumors and cells infected by viruses (Oldham, 1983). As NK cells have the capacity to detect changes in transformed cells in the absence of inflammatory signals, they can be responsible for tumor rejection in a direct fashion (Karre *et al.*, 1986). In the present investigation splenocytes obtained from mice treated (oral feeding) with non-irradiated fibroin and gamma-irradiated fibroin (150 kGy). Since NK cells suppress tumor growth and metastasis (Fuchs & Colonna, 2006), this study examined gamma-irradiatedfibroin effects on NK cell population. The population of NK cells evaluated by FACS for their content is shown in Fig. 14. This study was observed that as radiation dose increases, the population of NK cells is increased (irradiation dose dependent). The population of NK cells from 0 kGy and 150 kGy fibroin treated mice spleen were 104% and 117% respectively and the control group showed the lowest population content compare to 0 kGy and 150 kGy. This result supposes that gamma-irradiated fibroin enhance the content of NK cell population to inhibit tumor growth.

$CD4^+$ and $CD8^+$ T cells are the principal helper and effector cells, respectively, of adaptive cellular immunity, and many immunotherapy strategies are aimed at activating these cells to promote tumor cell destruction and long-term immune memory against recurrence of primary disease. Type 1 $CD4^+$ T cells (Th1) facilitate tissue destruction and tumor rejection by providing help to cytotoxic $CD8^+$ T cells (Suzanne, 2007).

The expression of $CD8^+$ and $CD4^+$ T cells in tumor-bearing mice (C57BL6) was evaluated by FACS for their content is shown in Fig. 15. As can be seen in Fig. 15, this investigation showed that the activation of $CD8^+$ T cells from 0 kGy and 150 kGy fibroin treated mice spleen were 15.96% and 20.34% respectively and in the case of $CD4^+$ T cells, the activation 28.6% and 31.0% was observed respectively. Both activations of $CD8^+$ and $CD4^+$ T cell on tumor only group showed lower than those of fibroin treated group.

These results indicate that gamma-irradiated fibroin enhanced NK cell and $CD4^+$

and $CD8^+$ T cell activity and NK cells induced from mouse spleen can be components of the innate immune system that interact with adaptive immunity through production of cytokines. It could be concluded that the gamma-irradiated fibroin might be a potential candidate as a value-added product in medical industry.

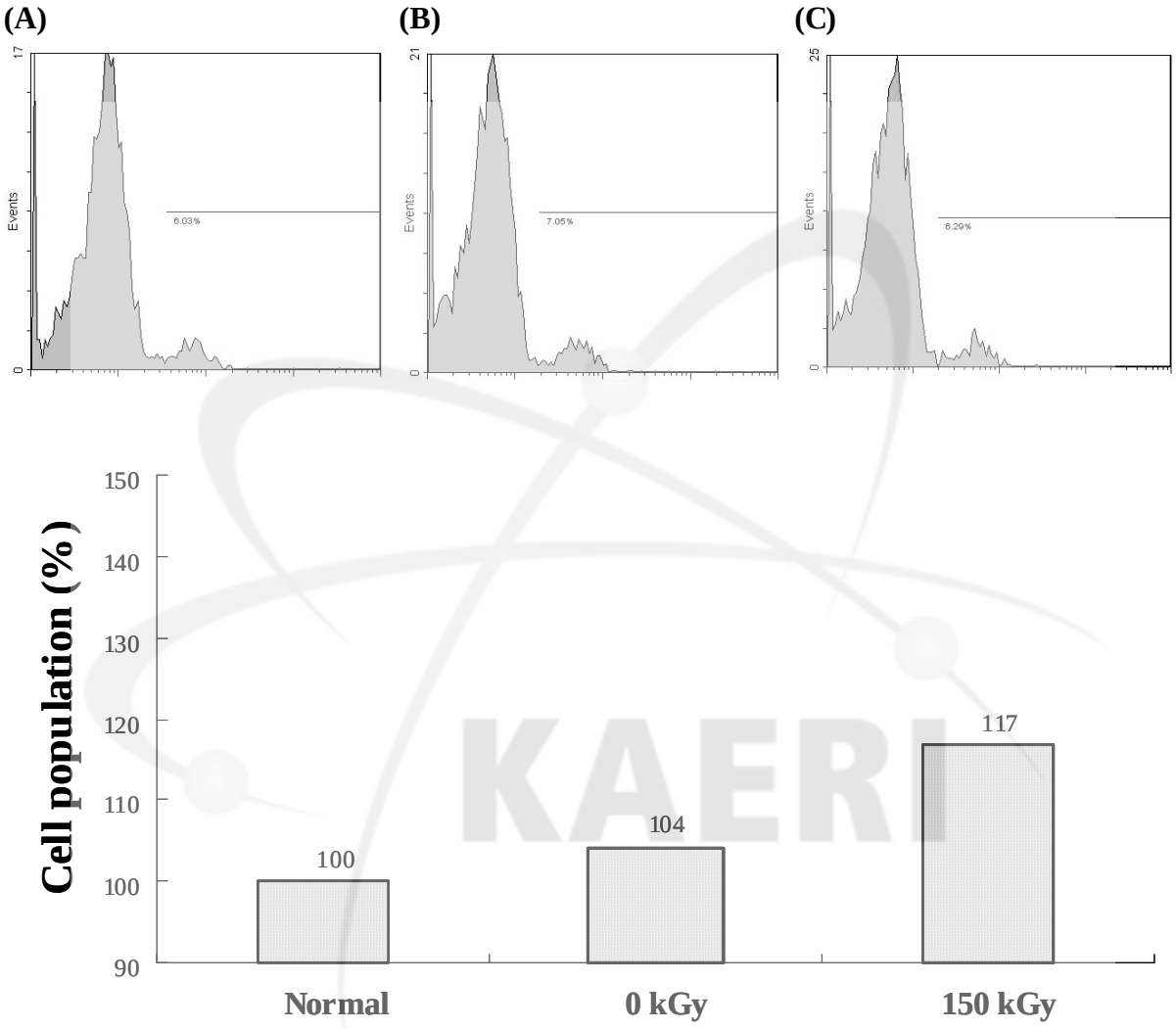

Fig. 14. NK cells were evaluated by FACS for their content. (A), Control cells incubated without fibroin; (B), non-irradiated fibroin (0 kGy); (C), irradiated fibroin (150 kGy).

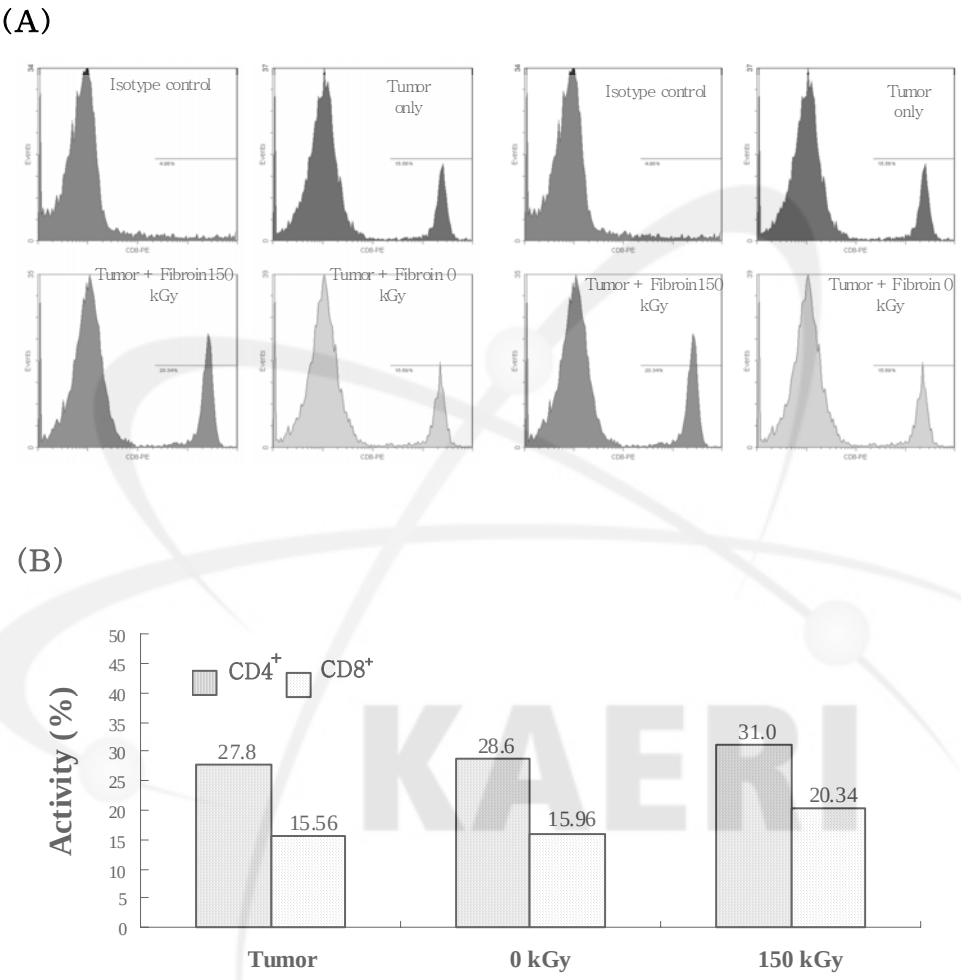

Fig. 15. Fibroin-treated mice were increased the expression of $CD8^+/CD4^+$ T-lymphocytes in tumor-bearing mice. (A), The numbers represent the percentages of $CD8^+$ cells; (B), The numbers represent the percentages of $CD4^+$ cells.

제2절 물성(점도/용해도) 및 기능성 증진된 저분자 베타글루칸 개발

1. INTRODUCTION

Gamma radiation is free of initiators and side products which result in chemically pure degradation product. Therefore, this technology is simpler and more environmentally friendly than conventional ones. A significant advantage in case of gamma irradiation is a final biological sterilization (Woods and Pikaev, 1994; Hugo, 1995) of irradiated materials that can be easily used for manufacturing biomedical products. Irradiation leads to the degradation of polysaccharides such as starch, cellulose, and pectin by complex mechanisms that have been difficult to elucidate (Ananthaswamy *et al.*, 1970; Charlesby, 1981; Deschreider, 1960). It is believed that degradation occurs largely by cleavage of the glycosidic bonds (Dauphin and Saint-Lébe, 1977; Sokhey and Hanna, 1993; Simic, 1983). Basic advantages of radiation degradation of polymers include ability to promote changes reproducibly and quantitatively, without the introduction of chemical reagents and without special needs to control for temperature, environment and additives (Charlesby, 1981).

β-glucan is a natural homopolysaccharide with high molecular weight and with high viscosity (Wood, 1986) and the polysaccharide is composed of glucose units linked together to form a long polymer chain and is a fiber-type of homopolysaccharide derived from the cell wall of yeast, oat and barley, and from many medicinal mushrooms (Yamada, 2000). β-glucans have been used in food industry as natural hydrocolloids due to their gelling capacity and ability to increase the viscosity of aqueous solutions (Dawkins and Nnanna, 1995). For many years β-glucan have been investigated for these immune enhancing properties that nutritionally potentiates and modulates the immune response (Falch *et al.*, 2000; Ross *et al.*, 1999). It is known that the immunomodulatory effects of β-glucans are influenced by their degree of branching, polymer lenghts, and tertiary structure, but there is still no consensus on the basic structural requirements for biological activity (Borchers *et al.*, 2004). High viscosity and low solubility of β-glucan make several difficulties to apply widely to food industries, because of retardation of malting, poor wort separation, difficulties in beer filtration, sauces, salad dressings, ice cream formulations and formation of undesirable precipitates (Carr *et al.*, 1990; Wood, 1986).

It has been reported that polymers can be degraded by acidic hydrolysis (Hasegawa *et al.*, 1993 Bohn and BeMiller, 1995) or enzymatic treatment (Ilyina *et al.*, 2000; Shimokawa *et al.*, 1996). However, in these methods further purification is necessary due to the presence of additives used to initiate reactions and the formation of side products. Even though several methods such sulfation, phosphation, and carboxymethylation, acid and alkaline hydrolysis, enzymatic digestion, and ultrasound irradiation have been applied for depolimerization of β-glucan, ionizing radiation with high energy has not been conducted to the research of β-glucan. Hence the aim of the present study is to apply radiation technology to β-glucan in order to reduce the molecular weight and viscosity and to increase its solubility in water.

2. MATERIALS AND METHODS

Materials

β-glucan sample was purchased from ACE BIOTECH (Chungbuk, Korea). Content of total polysaccharide is 97.2% determined by Phenol-sulfuric acid method (Masuko *et al.*, 2005) and content of β-glucan is about 80% by Laminarinase method (Dubois *et al.*, 1956). Powdered β-glucan was dissolved in deionized water in the concentration of 100 ㎎/㎖ (10%, W/V), and the solution was gamma-irradiated, and stored at 4℃ until use a subsequent experiments.

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Gamma irradiation

Gamma irradiation was performed at a cobalt-60 irradiator (point source AECL, IR-79, MDS Nordion International Co., Ltd, Ottawa, Ontario, Canada) in the Advanced Radiation Technology Institute, the Korea Atomic Energy Research Institute (Jeoung-Eup, Korea). The source strength was approximately 300 kCi with a dose rate of 10 kGy/h and temperature of irradiation room was 17℃. Dosimetry was performed using 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). The dosimeters were calibrated against an international standard set by the International Atomic Energy Agency (Vienna, Austria). The

applied doses in this study were 0, 10, 30 and 50 kGy at 17℃.

Monitoring the changes of molecular weight by gel permeation chromatography

Gel permeation chromatography (GPC) was conducted to monitor the changes of the average molecular weight of β-glucan by gamma irradiation. GPC was performed by Waters GPC system equipped with Waters 515 pump, 2 × PLaqagel OH Mixed (7.8 × 300mm) column and Waters 2410 refractive index detector. Each sample solution was 10 fold-diluted with distilled water for making a concentration of 10 ㎎/㎖, and 100 ㎕ of the diluent was loaded in GPC system. The column was operated at 40℃ and eluted with distilled water at a flow rate of 1.0 ㎖/min. The column was calibrated by various standard dextrans at a concentration of 0.1% w/w. Analysis was used as Millennium 3.05.01 (Waters, Milford, MA).

Measurement of viscosity and solubility

The viscosity of β-glucan sample solutions was measured with Brookfield viscometer (DV-II+ pro, Brook-field Engineering Laboratories, MA, USA) using spindle S21 at 180 rpm at room temperature.

For determinating the differences of solubility by irradiation treatment on β-glucan, samples solutions were lyophilized. Each sample powder of 2 g was put in a glass tube (50 ㎖) with a cap, voltexed with deionized water of 10 ㎖ for 20 min, and centrifuged at 3500 × *g* for 20 min using a centrifuger (Hitachi, Tokyo, Japan). The supernatant was separated and was dried at 100℃ for 2 h using a dryer (Sanyo, Osaka, Japan), and the weight of dried products from the supernatant was determined. The solubility was calculated as follows;

$$\% \text{ Solubility} = [\text{g of dried supernatant}]/[\text{g of initial powder } \beta\text{-glucan}] \times 100$$

Secondary structure by fourier transform infrared spectroscopy

Application of Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy has been shown to be a useful tool in monitoring structural changes in biopolymers

(Wilson *et al.*, 1988; Sartori *et al.*, 1997). For observing whether the structural changes by irradition were only occurred from the cleavege of glycosidic bond or affected to the functional properties, FTIR spectra were taken on Shimazu FTIR 8100A spectrophotometer (Shimazu, Kyoto, Japan) combined with computer system Shimazu DR 8030 at the wavelength region between 4000 and 500 cm^{-1}. Samples were prepared as a thin film mixed with KBr at polymer/KBr w/w ratio 1-100. Obtained spectra were the result of 24 scans at the spectrophotometer resolution 8 cm^{-1}.

Surface structural analysis by scanning electron microscope

By the method of Sokhey and Hanna (1993), the microstructural changes of β-glucan by gamma irradiation was observed by the scanning electron microscope. Samples were fixed on a cylindrical microscope stub covered with carbon strip and coated with a thin layer of gold, followed by observation under a scanning electron microscope (JEOL, Tokyo, Japan). A magnification of 140 was used.

Measurement of color

The color of the gamma irradiated β-glucan was measured the values of L^* (lightness), a^* (redness) and b^* (yellowness) by a Hunter spectrophotometer (Spectrophotometer CM-3500d, Minolta Co., Osaka, Japan). The instrument was calibrated to standard black and white tiles before an analysis. Color measurement was triplicated through a computerized system by using Spectra Magic software (version 2.11, Minolta Cyberchrom Inc. Osaka, Japan).

Analysis of microorganism

The samples (1 ㎖) was blended with steriled 0.1% (W/V) peptone water (9 ㎖) (Difco Laboratories, Detroit, MI, USA) using a mixer for 30 sec. Total aerobic bacterial, yest and mold were obtained from plate count agar (Difco Laboratories, Detroit, MI) and a potato dextrose agar (Difco, Laboratories, Sparks, MD, USA). The plates were incubated at 37℃ for 28 h (Total aerobic bacteria) or at 25℃ for 48

h (yest and mold), and the colony forming units (CFU) per gram were counted at a dilution of 30 to 300 CFU per plate. Experiments with each bacteria culture were independently conducted twice.

Change of reducing power

Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNSA) method (Miller, G. L. 1959). One milliliter of the sample was transferred into 15 ㎖ glass tubes and 2 ㎖ of the modified DNSA regent (0.5 g dinitrosalicylic acid, 8 g sodium hydrate and 150 g rochell salt in distilled water up to 500 ㎖) was added. The solution was mixed with a vortex mixer for 5 sec and was boiled for 10 min, and then cooled in ice. The reducing sugar level of the sample was analyzed by a spectrophotometer (UV-1601 PC, Shimadzu Co., Tokyo, Japan) in reference to the glucose standard.

UV-VIS absorption sprectroscopy

UV scanning of the irradiated β-glucan solutions (concentration of 1% w/w) was measured at the range from 200 to 500 nm in a spectrophotometer (UV-1601 PC, Shimadzu Co., Tokyo, Japan) using the same non-irradiated β-glucan solution as a reference.

Enzymatic hydrolysis of β-glucan

β-D-glucans assay kit is purchased from megazyme international ireland Ltd. (Wicklow, Ireland). β-D-glucans were solubilised in concentrated (37%; 10 N) hydrochloric acid and then extensively hydrolysed by 1.3 N HCl at 100℃ for 2 h. Hydrolysis to D-glucose was completed by incubation with a mixture of highly purified enzymatic kits. While some β-glucans are readily soluble in hot water or hot KOH, these solvents are not effective in solubilising the β-glucans from yeast. Analysis of these glucans requires prior partial acid hydrolysis to remove gel-forming properties and covalent links to other polysaccharides. Measure the absorbance of all solutions at 510 nm against the reagent blank.

Experimental animals

BALB/c mice (6-7 weeks) were purchased from the Orient Inc. (Charles River Technology, Seoul, Korea). The mice were housed in polycarbonate cage and fed with a standard animal diet and water *ad libtum* under controlled temperature conditions (22±2℃, 8℃) with 12 hr light and dark cycles. The animals were divided into five groups (n=6) normal control, mice received 0 kGy, 10 kGy, 30 kGy, and 50 kGy β-glucan for a period of 7 days (24 hrs interval) at a concentration of 50 ㎎/㎏ body wt. by oral gavage (0.5 ㎖ volume). After the experiment period animals were sacrificed under ether anesthesia by cervical decapitation and spleen was dissected and immediately maintained in RPMI-1640 medium (Sigma). Blood collected was stored at -80℃ for HDL and LDL assay. Animal research procedures were conducted according to principles enunciated in the 'Animal Care Act', prepared by the Ministry of Agriculture and Forestry, Korea.

Isolation of splenocytes from animals

Spleen cells were obtained by gentle disruption of the spleen (BALB/c mice) and repeated pipetting was done to attain single cell suspension. After the cells were twice washed in ice cold RPMI-1640 medium developed by Moore *et al.* (1985) at Roswell Park Memorial Institute, lymphocytes were separated from erythrocytes using ice cold distilled water for 30 s and then counted for cell viability (90%). Splenocytes were maintained in RPMI-1640 medium with 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin at 37℃ under 5% CO_2 in air (Cederbrant K, 2003).

Spleen cell culture

Spleen cell suspensions were plated in 96-well tissue culture plates at a final concentration of 1×10^6 cells/well maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/㎖ penicillin and 100 U/㎖ streptomycin and then cultured at 37℃ in 5% CO_2. The cells were stimulated with β-glucan at a final concentration of 2.5 ㎍/㎖ with mitogen Con A (1 ㎍/㎖) and normal without mitogen. Cultured

supernatant harvested after 24 h and stored at -70℃ for cytokine evaluation.

Cell proliferation assay

Spleen cell proliferation was detected by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tertrazolium bromide) assay method. Following an incubation of the cells with the β-glucan for 24 h, 30 ㎕ of 5 ㎎/㎖ MTT in PBS was added to each well and the plate was incubated at 37℃ for 2 h. The plate was then centrifuged and the medium was removed. Then formazan crystals were dissolved using 100 ㎕ of dimethylsulfoxide (Sigma, St. Louis. Mo). After incubation at 37℃ for 5 min, the optical density was measured at 595 nm in automated microplate ELISA reader.

Cytokines assay

Supernatants cultured for 24 h were evaluated by BD OptEIA^{TM} set by following the manufacturer's instruction (BD Biosciences, San Jose, CA). Cytokines Sandwich enzyme-linked immunosorbent assay (ELISA) for IL-2, IFN-gamma was carried out in duplicate using 96-well ELISA plates (costar 3595, Corning, NY) coated with 100 ㎕ aliquots each of anti-mouse IL-2, IFN-gamma monoclonal antibodies at 1.0 ㎎/㎖ in PBS at pH 7.4 and incubated overnight at 4℃. The plates were washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% $NaNO_3$ for 1 h. After additional washing, the sample and IL-2, IFN-gamma standards were added and incubated at 37℃ for 2 h. After 2 h incubation at 37℃, the wells were washed and then 0.2 ㎎/㎖ each of biotinylated anti-mouse IL-2, IFN-gamma were added and again incubated at 37℃ for 1 h. After washing the wells, avidin-peroxidase was added and the plates were incubated for 30 min at 37℃. Wells were again washed and TMB substrate was added. This reaction was stopped by 1 N HCl. Color development was measured at 595 nm in automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant IL-2, IFN-gamma in serial dilutions

Measurement of free radical scavenging activity (DPPH assay)

The free radical scavenging activity using the 2,2-diphenyl-1-picryl-hydrazil (DPPH) reagent was measured by a method described by Amarowicz *et al.* (2004) with a slight modification. To 0.5 ㎖ of β-glucan dissolved in distilled water (2.5, 5 and 10 ㎎/㎖) freshly prepared DPPH radical in a ethanol solution (0.1 mmol) was added and vortexed. Reaction mixture was incubated for 25 min at room temperature in dark then centrifuged at 6400 rpm for 5 min. All aliquots in triplicate were read at 517 nm using automated microplate reader. The radical scavenging inhibition concentration was determined and compared with the corresponding reference compound BHT (1, 0.5, 0.25, 0.125, and 0.0625 ㎎/㎖ in EtOH)

Blood cholesterol (HDL&LDL) assay

The level of blood cholesterol High density lipoprotein (HDL) and low density lipoprotein (LDL) were examined by autoanalyser (Hitachi, Japan 7180) a medium-sized biochemistry automatic analyzer with user interface featuring operability unique to a general-purpose OS (Windows NT(R)) with touch screen. HDL and LDL calibrator for (lipids) automated system were used (Roche, USA). Mice body weight and spleen weight were recorded before and after β-glucan adminstration on day 1 and 7.

3. RESULTS AND DISCUSSION

Changes of molecular weight by gamma irradiation

Fig. 16 shows the modification of molecular weight of β-glucan by high dose irradiation as an expression of radiation effect under different doses. The molecular weight of β-glucan in solution was found to be 178 kDa in non-irradiated sample, while a considerable decrease in the molecular weight was observed in the gamma-irradiated β-glucan samples, and molecular weights of samples irradiated with 10, 30 and 50 kGy were about 62 kDa, 32 kDa and 25 kDa, respectively. It has been reported that β-glucan with > 100 kDa of molecular weight are considered as

high molecular weight and low molecular weight is < 50 kDa (Bohn and BeMiller, 1995). These changes occurred by irradiation may be due to the alterations in the functional group of the native molecule which may involve breakage of glycosidic bond of polysaccaride (Sokhey and Hanna, 1993; Simic, 1983). This result was well supported by the reports related with the depolymerization of polysaccharides by radiolysis on starch (Wu *et al.*, 2002; Raffi *et al.*, 1981), chitosan (Ilyian, 2000), guar gum (Harding and Mitchell, 1996) and alginate (Wasikiewicz, 2005; Nagasawa *et al.*, 2000).

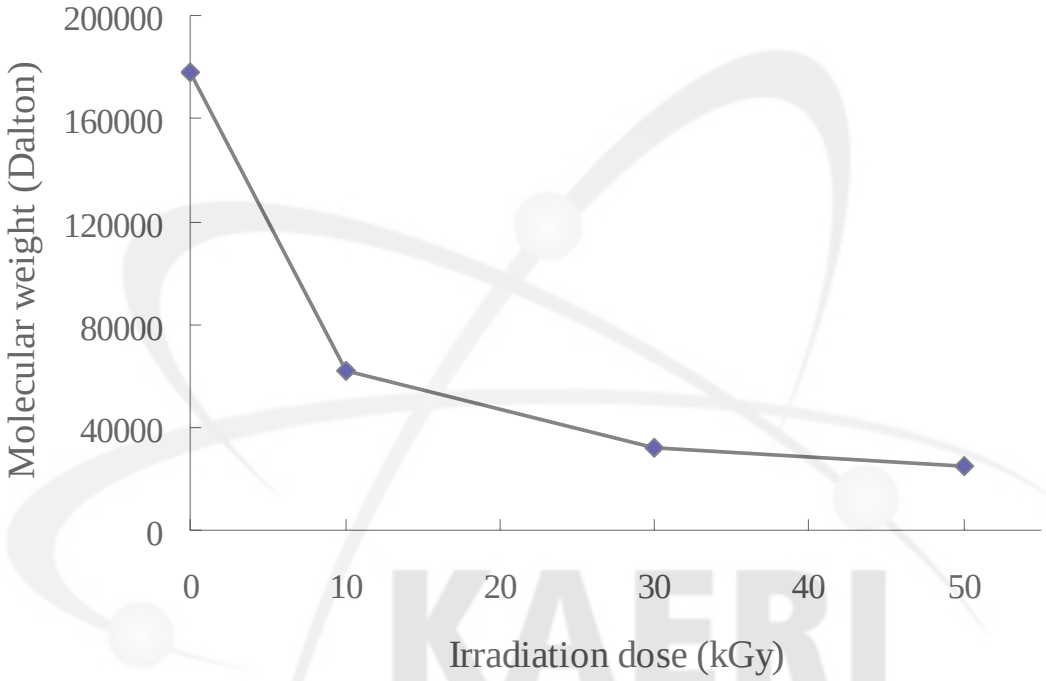

Fig. 16. The effect of irradiation dose on the molecular weight of β-glucan.

Changes of viscosity and solubility in water

Fig. 17 and 18 show the changes of the viscosity and solubility of β-glucan solution by the gamma irradiation, respectively. As the absorbed dose increased, the β-glucan solution showed significant decrease of viscosity. The viscosity of non-irradiated sample was 191.93 cp, while the viscosity of 50 kGy-irradiated sample appeared to be 43.9 cp with the decrease rate of about 73% compared with non-irradiated one. The decrease of the viscosity was occurred by the radiolysis of β-glucan. This results were well explained in other studies on starch, agar, alginate, carageenan, etc (Graham *et al.*, 2002; Deschredier, 1960; Aliste *et al.*, 2000).

Water solubility of β-glucan increased upon the increase of the absorbed dose of gamma radiation. In general, β-glucan can be disolved in water till about 50% (51.38 % in this study), while the solubility appeared to about 55.76%, 75.81% and 81.72% in 10 kGy-irradiated, 30 kGy-irradiated and 50 kGy-irradiated samples, respectively. The increasing rate of solubility could be calculated as 0.65% per increasing dose of 1 kGy within R^2 = 0.9499. The increase of solubility of β-glucan by the gamma irradiation might have augmented by the modification and degradation of the amylose and amylopectin structures with the low molecular weight fractions (Graham *et al.*, 2002). This result can be explained by the reason that the breakdown and depolymerization of β-glucan by radiolysis with high energy induce the production of the smaller fragments and the low molecular fraction to be dissolved in water more easily (MacArthur and D'Appolonia, 1984).

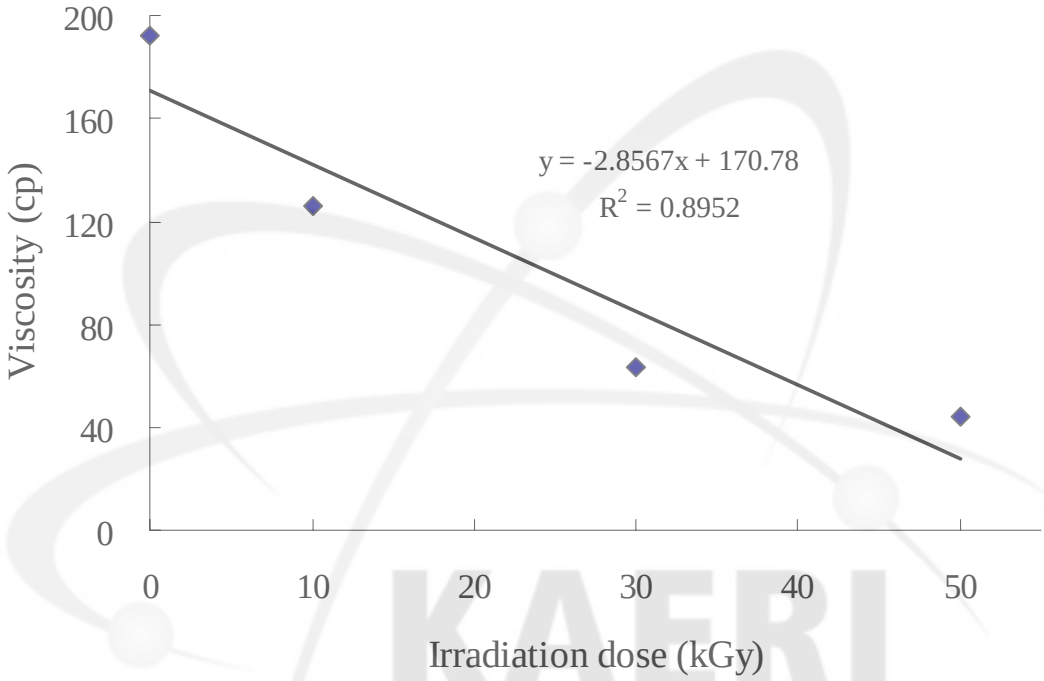

Fig. 17. The changes of viscosity of β-glucan irradiated at various doses.

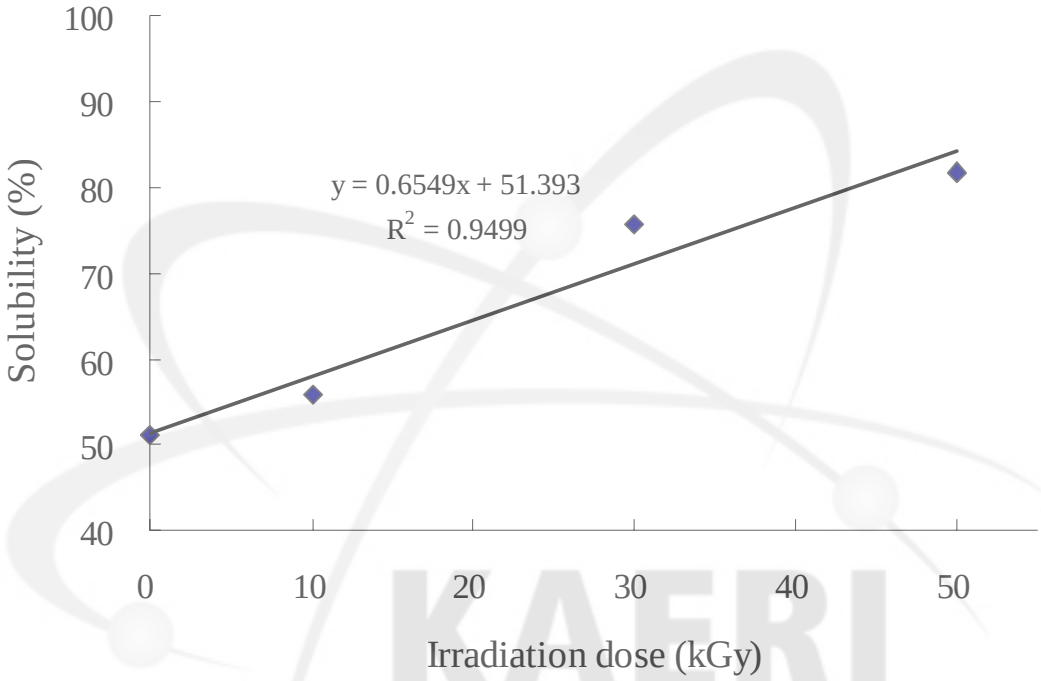

Fig. 18. The effect of irradiation on water solubility of β-glucan at various doses.

Analysis of FTIR spectra

In spite of the fact that on the one hand the infrared characteristics of glucose are well known (Mathlouthi and Koenig, 1986), on the other hand, information about glucans is rather insufficient. β-glucan is assumed that deformation may occur due to splintering of glycosidic bonds (von Sonntag, 1980). To confirm this assumption, β-glucan was subjected to FTIR analysis before and after irradiation. Fig. 19 illustrates FTIR spectra of β-glucan treated under different doses of gamma radiation. In the region of functional group of infrared spectra, there are noteworthy absorptions at 3415, 2929, 1748 and 890 cm^{-1}, which corresponds to the stretching absorption bands of poly -OH, C-H, C=O and β-configuration, respectively. The C-O vibrations ranging at 1200 - 950 cm^{-1} as well as in the anomeric region at 950 - 750 cm^{-1} distinguished β-D-glucan from a-D-glucan spectra (Mathlouthi and Koenig, 1986; Gutierrez *et al.*, 1996). This observation showed that the gamma-irradiated β-glucan molecule and the native compound had similar pattern of FTIR spectra without any notable changes in the functional group status.

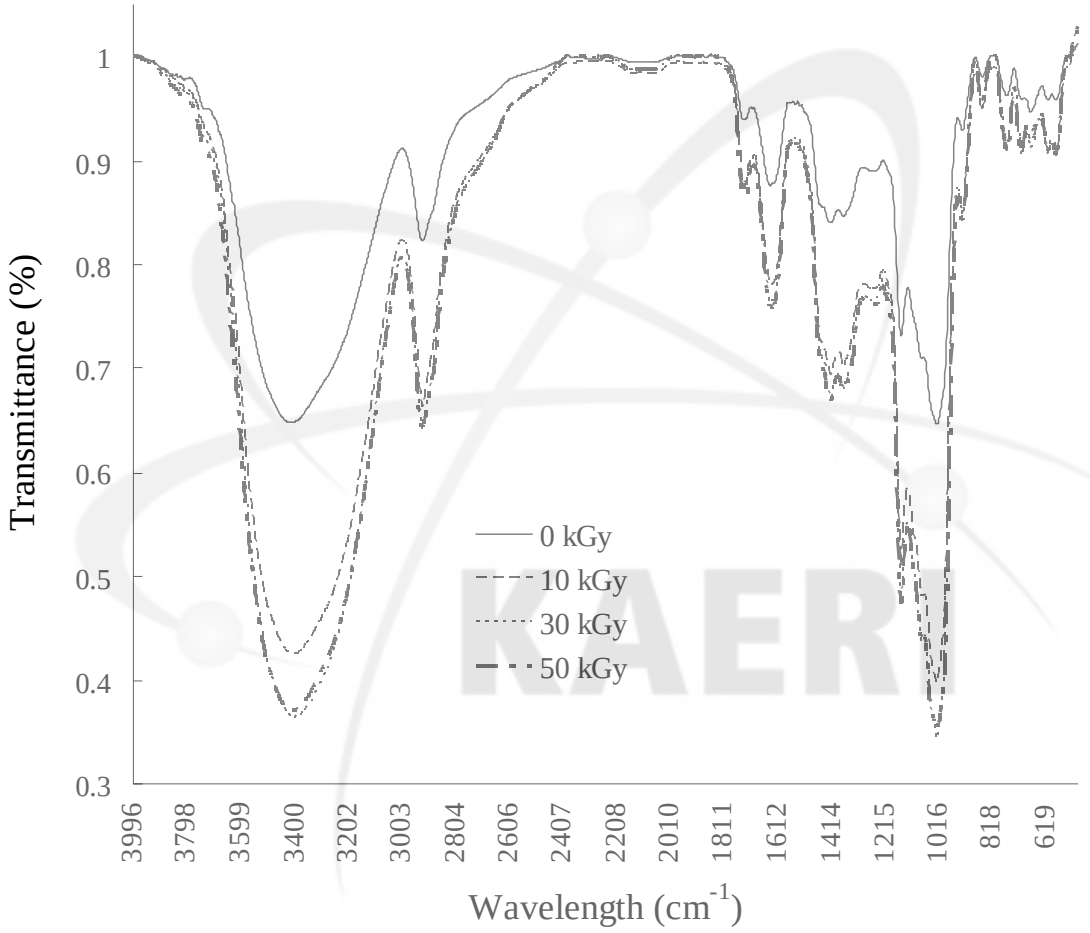

Fig. 19. FTIR spectra of β-glucan irradiated at various dose; (——) 0 kGy, (- - -) 10 kGy, (· · · ·) 30 kGy and (— - ·) 50 kGy irradiation dose.

Imaging with scanning electron microscopy

Microphotographs of β-glucan granules obtained from non-irradiated and 50 kGy-irradiated β-glucans using scanning electron microscopy (SEM) at 140 magnification for evidence of β-glucan granular fissures or splitting (Fig. 20). Sokhey and Hanna (1993) stated that the granule structure remains visually undamaged at low irradiation dosages, but may cause severe damage at higher dosages. Wu *et al.* (2002) reported that gamma irradiation even at 1 kGy affected the starch granule structure and the shape of the starch granule was deformed by gamma irradiation. It was observed that β-glucan granules were deformed and smaller granules generated in irradiation samples compared to non-irradiated ones. The 30 kGy-treated β-glucan showed moderate changes whereas the 50 kGy-treated β-glucan showed the higher deformed granules when compared with non-irradiated β-glucan. Hence these fissures and splitting of β-glucan granules were caused mainly due to breakdown of glycosidic bonds by gamma irradiation.

Until today acid treatment and enzymatic hydrolysis are being used to resolve the problem of high molecular weight compounds. Even though these methods are effective in decreasing the molecular weight, still they found to have certain disadvantages such as disorder of triple helical structure of the β-glucan and fall short to hydrolyse the highly β-1,6-branched β-1,3-glucans efficiently (Shin and Lee, 2003). The methods for lowering the molecular levels of polysaccharide has been proposed and the hydrolysis of polysaccharide using heat, chemical or enzymatic methods has been applied presently (Cho *et al.*, 2003). Chemical treatment is a very common and fast method but it has some defects, such as high cost, low yield, and acidic wastes by the use of HCl (Jeon and Kim, 2002). The enzymatic processes are generally preferable over chemical reaction because the hydrolysis can be controlled more easily, but oligomers are not obtained in a good yield (Marie *et al.*, 1994). Also, the method of enzymatic hydrolysis has a long processing time and very sensitive to pH change, and addition of chemical or enzymes makes it difficult for industrial mass production.

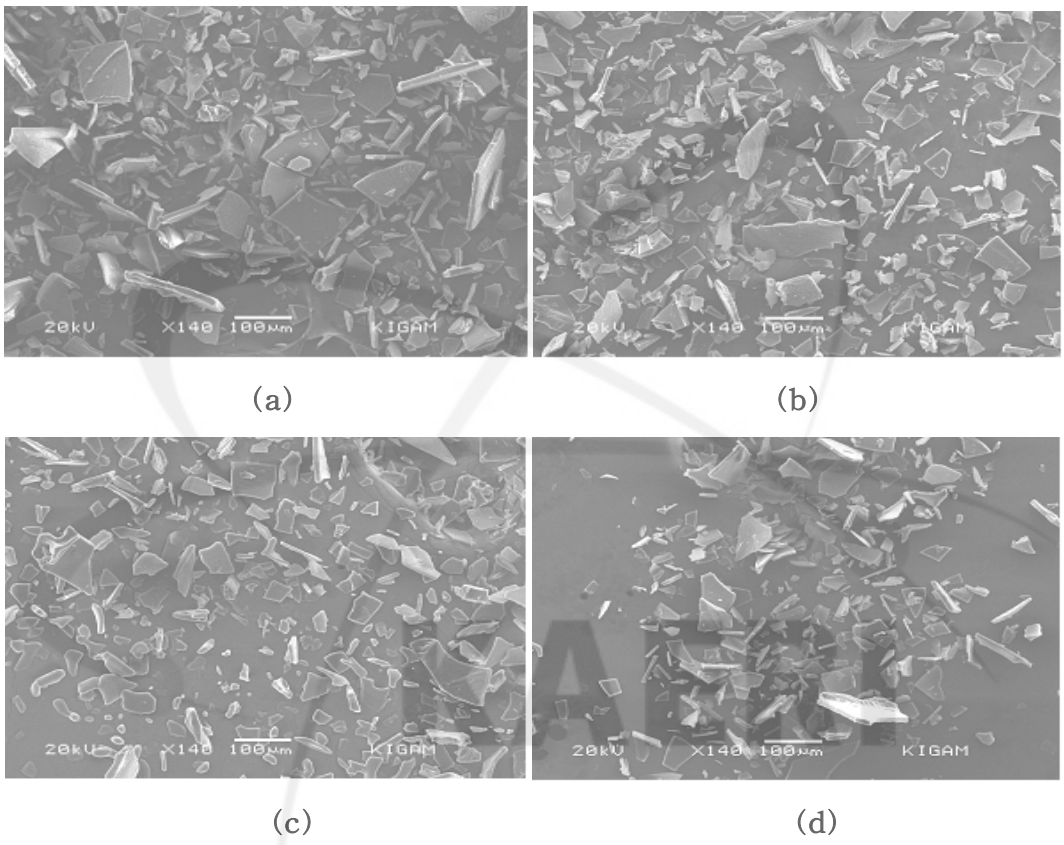

Fig. 20. Scanning electron microscopy (SEM) images of β-glucan irradiated at different dose; (a) control, (b) 10 kGy-irradiated, (c) 30 kGy-irradiated and (d) 50 kGy-irradiated.

Change of color

The color of β-glucan is used as an important index to determine the external quality of β-glucan. Table 2 shows the color of β-glucan changed by the gamma irradiation. β-glucan exhibited the significantly decreasing tendencies in lightness with the increase of the dose, and the values of a^* (redness) and b^* (yellowness) of β-glucan showed the increasing tendencies. These increases were affected by the products produced as a result of the non-enzymatic browning reaction, and this reaction was catalyzed by irradiation, which are corresponding to other researches (Rombo *et al.*, 2001; Wootton *et al.*, 1998 ; Roushdi *et al.*, 1981; Fan *et al.*, 2002). Some researchers observed that color intensity of the irradiated polysaccharides with increased irradiation (Roushdi, *et al.*, 1981; Lee *et al.*, 2004). Nicoli, Casadei, Guerzoni, and Lerici (1994) suggested that irradiation leads to non-enzymatic browning reactions similar to those induced in heat treatment food.

Table 2. Effect of gamma irradiation on Hunter color values of β-glucan

Irradiation dose (kGy)	L^*	a^*	b^*
0	$89.26^{a1)}$	-0.45^{f}	5.16^{g}
10	88.05^{b}	-0.06^{e}	6.64^{f}
30	86.70^{c}	0.37^{d}	8.21^{e}
50	86.18^{d}	0.54^{c}	8.92^{d}

1) Different letters ($^{a-g}$) within a same column differ significantly ($p < 0.05$).

Microbial analysis

Table 3 shows the number of total aerobic bacteria, yeast and mold of a irradiated β-glucan solution by the gamma irradiation. Yeast and mold was not detected in non-irradiated and irradiated β-glucan. However, β-glucan tested showed microbial contamination by being detected the total aerobic bacteria ranged about 2-4 Log CFU/g. The result of the study showed that total aerobic bacteria of control was the highest and followed by 10, 30, and 50 kGy. Gamma-irradiation showed some positive effect on the reduction of microbial contamination in β-glucan, where microorganisms were not detected in 50 kGy irradiated β-glucan. The reason of the decrease of the microbial contamination might be due to improved anti-microbial property of irradiated β-glucan against microorganisms. Thus Irradiation is effective in reducing microorganisms, likewise a good method used for inactivating pathogens in food materials (Adeil Pietranera, 2003; Abu-Tarboush, 1996; Chawla, 2003). In addition, irradiation processing of food has already proved to be safe and effective means of reducing or eliminating biological hazards in food (WHO, 1999). Therefore, the study propose irradiation method could be used as useful technique to improve the anti-microbial property β-glucan against microbial contamination.

Table 3. Effect of gamma irradiation on the total aerobic bacteria and yeast mold β-glucan

Irradiation dose (kGy)	Aerobic bacteria	Yeasts mold
0	4.71 ± 0.05[1)]	ND[2)]
10	2.08 ± 0.05	ND
30	1.24 ± 0.34	ND
50	ND	ND

1) Mean ± standard deviation (n=2).

2) Viable colony was not detected at detection limit < 10^1 CFU/g.

Reducing sugar

The changes of the reducing sugar content in β-glucan by the gamma irradiation tend to increase in proportion to the dose as shown in Fig. 21. The rate of increase of the reducing sugar in β-glucan were 0.027%. The β-glucan content of reducing sugar (non-irradiated) group were 0.917%, while in lower doses of irradiation 10 and 30 kGy showed considerable increase in the reducing sugar content about 1.128% and 1.973% respectively. β-glucan with 50 kGy of gamma ray was increased to 2.173% compared to control and lower doses of irradiation. The R^2 values of the β-glucan were 0.9383, and showed linear curves with the function of the reducing sugar content and irradiation dose. The fact that the reducing sugar contents were increased in proportion to irradiation dose consistent with other studies (Raffi *et al.*, 1981; Ananthaswamy *et al.*, 1970). The increase of the reducing sugar content was caused by the production of several low molecular weight sugars such as glucose by depolymerization. The increase in irradiation dose is directly proportional to the depolymerization (Ananthaswamy *et al.*, 1970) whereas irradiation induced scission of the glycosidic linkage may lead to the generation of a radical at the C1 position on the glucose molecule or disaccharide in the presence of water, and consequently, these radiolytic end products had a reducing power (Raffi *et al.*, 1981; Molins, 2001). Thus, the reducing sugar produced catalyzed the non-enzymatic browning reactions, which influenced the color of β-glucan showing the increase in yellowness as seen in Table 2.

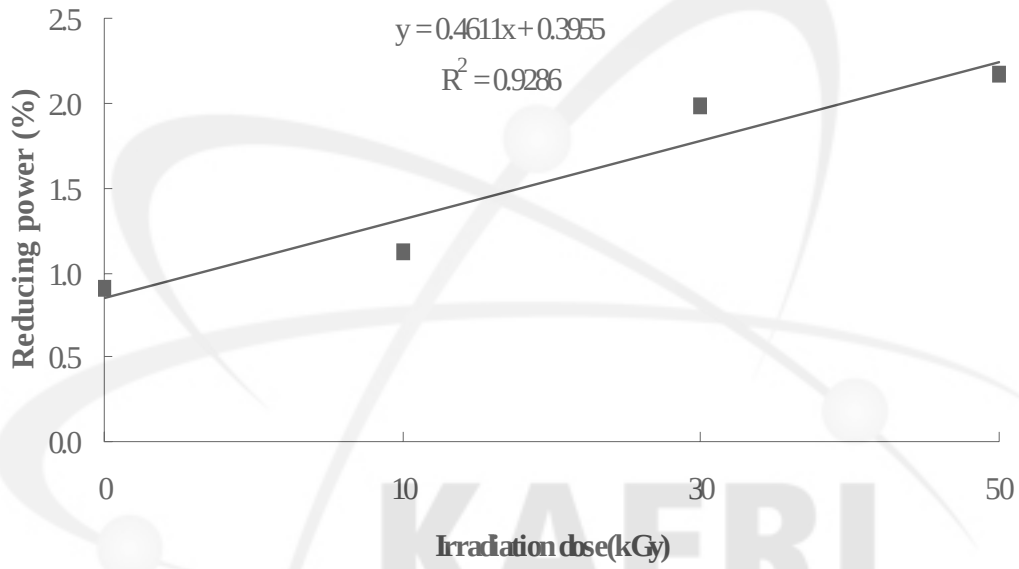

Fig. 21. Changes of the reducing level (%) in the β-glucan solutions (1.0 M) after gamma-irradiation. Irradiation: the sugar solutions were irradiated at 0, 10, 30, 50 kGy (n=3).

Change of UV-VIS absorption pattern of β-glucan by gamma irradiation

UV absorption spectra of irradiated β-glucan was examined and results are shown in Fig. 22. In UV absorption spectra of β-glucan at 265 nm was increased by the gamma irradiation. Reports explained (Nagasawa *et al*., 2000) that above absorbance peak can be ascribed to double bonds formed after the main chain scission of the polymer followed by the gamma irradiation. Whereas UV absorption spectra of β-glucan at peaks between 250 and 280 nm were also observed. It is consistent with previous results (Ulanski and Rosiak, 1992), that observed peaks were describe carbonyl and carboxyl groups. Therefore, from this data we reinforce that the decrease in the molecular weight and viscosity could have resulted from chain scission of the polymer by the gamma irradiation as compared in Fig. 19.

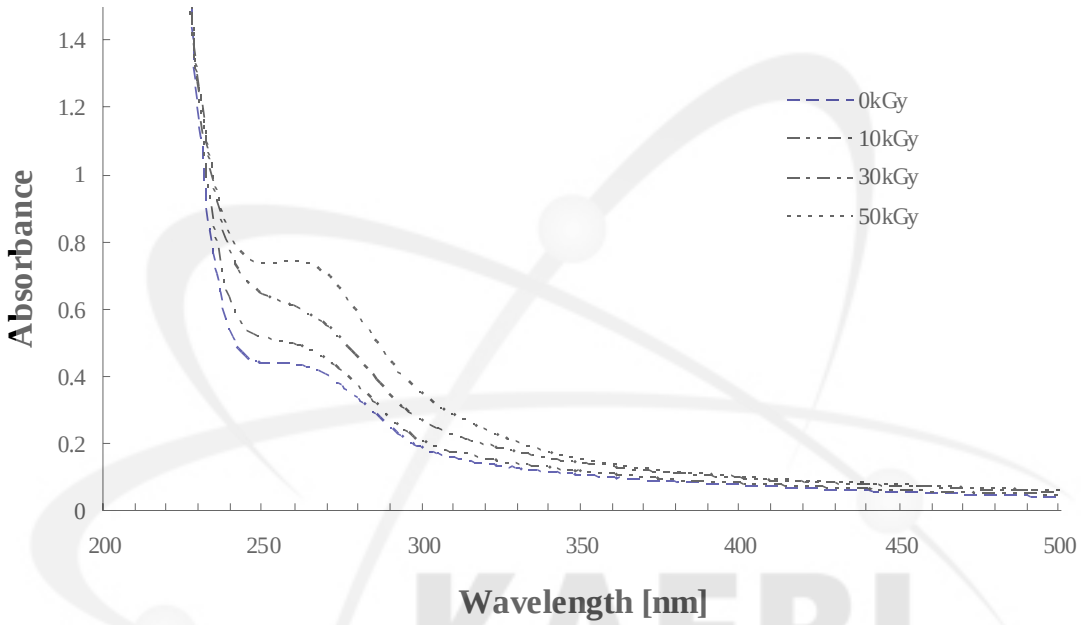

Fig. 22. Absorption spectra of β-glucan aqueous solution (concentration of 1% w/w) irradiated at different gamma-irradiation doses.

Linkage structure in β-glucan

A commonly used method for analysis of the structure of β-glucan is hydrolysis with a specific β-glucanase enzyme (Johansson *et al.*, 2000; Wood, *et al.*, 1991). Table 4 shows the changes of contents of β-1,3-glucan, β-1,4-glucan and β-1,6-glucan by the gamma irradiation. As the irradiation dose was increased, the contents of β-1,3-glucan, β-1,4-glucan and β-1,6-glucan were decreased. The content of total β-glucan of non-irradiated sample was 83.04%, while those of 10, 30 and 50 kGy irradiated sample were considerably decreased at 70.14%, 60.43% and 56.80% respectively. The reason of the decrease of the β-glucan content is assumed that deformation of β-glucan could have occoured due to splintering of glycosidic bonds by gamma irradiation. Many reports suggested that gamma irradiation was mainly breakage of glycosidic bonds of polysaccharide (Von Sonntag, 1980). Gamma irradiation might have induced random splinteringand breakage in all structure of β-glucan such as β-1,3-glucan, β-1,4-glucan and β-1,6-glucan.

Table 4. Changes of contents of irradiated β-glucan solution by the gamma irradiation.

Irradiation Dose (kGy)	β-glucan (%)			Total (%)
	β-1,3-glucan	β-1,4-glucan	β-1,6-glucan	
0	35.79	36.24	11.01	83.04
10	30.21	30.43	9.50	70.14
30	25.96	25.81	8.66	60.43
50	24.88	24.14	7.78	56.80

Proliferative index of spleen by non - irradiated and irradiated β-glucan

In vitro and mammalian studies has demonstrated the non-specific immune-enhancing effects of yeast β-glucan (Babineau *et al.*, 1994). Our study aimed to examine the spleen cell proliferation of non - irradiated and irradiated β -glucan *in vitro*. Murine spleen cells were used to evaluate the immunomodulatory activity of irradiated and non-irradiated beta glucan. The rate of spleen cell proliferation (Fig. 23) was measured by MTT assay after 24 h stimulation of splenocytes with β-glucan (2.5 ㎍/㎖ non - irradiated and irradiated and T cell mitogen Con A (1 ㎍/㎖). The results showed that when 2.5 ㎍/㎖ of irradiated and non-irradiated β-glucan was used, Con A stimulated splenocytes proliferation was enhanced while non-stimulated splenocytes were not affected. Splenocytes proliferative optical density average mean ± SD of non - irradiated β-glucan with mitogen was 0.310 ± 0.015 (stimulatory percentage 122%) while irradiated β-glucan with mitogen also showed sensible increase ($p < 0.05$) in the spleen cell proliferation, 0.328 ± 0.007, 0.345 ± 0.012 and 0.361 ± 0.005 (stimulatory percentages between 130 ~ 143%) with the increase in radiation dose from 10, 30 and 50 kGy respectively. The spleen cells stimulated with 50 kGy irradiated β-glucan showed significant ($p < 0.05$) proliferative index compared to non-irradiated β-glucan. A study has argued that β -glucan molecular weight ranging from 1 kDa ~ 30 kDa may display better immunmodulating effect compared to intact high moleular β-glucan in this view point our *in vitro* results disclose that deformation of β-glucan under safe irradiation dose could act *in vitro* on the regulation of cellular immunity inducing proliferation of lymphocytes (Cristina *et al.*, 2005; Lehmann *et al.*, 2000).

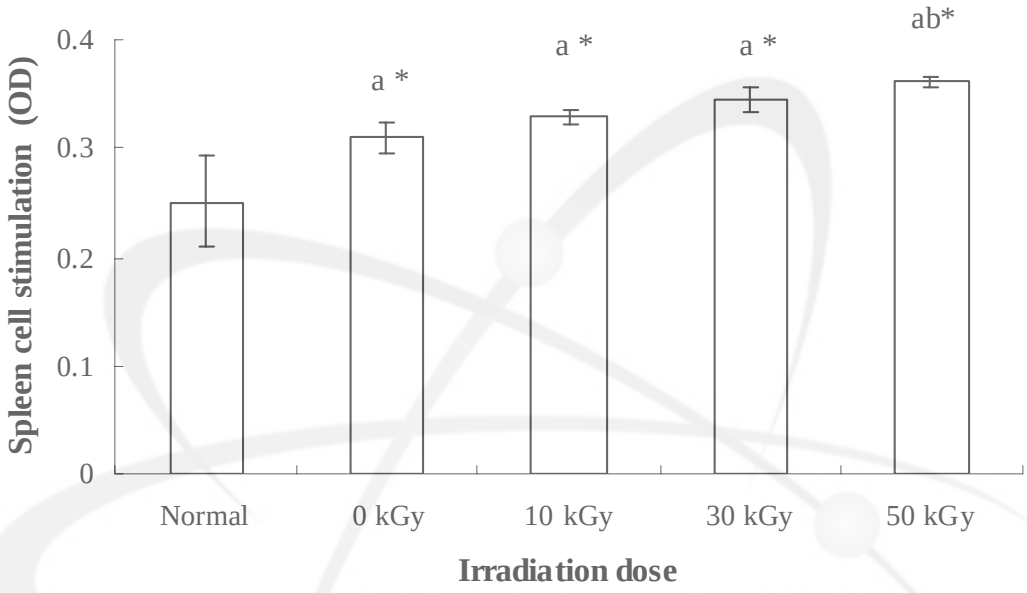

Fig. 23. Effects of β-glucan (non-irradiated and irradiated) on mouse spleen cell proliferation (normal spontaneous stimulation and Con A stimulated proliferation with β-glucan (non-irradiated and irradiated).

Effect of irradiated β-glucan on spleen cell cytokine release

It is well-known that IFN-gamma, IL-2 and TNF-α are key cytokines in macrophage activation, decisive for devastating different infectious agents by immunoediting mechanism (Leenen *et al.*, 1994). T cells (cytotoxic and Th1) and natural killer (NK) cells secrete IFN-gamma that can modulate natural and specific immune responses. In order to investigate whether the irradiated and non-irradiated β-glucan could increase Con A-induced spleen cell Th1 associated cytokines release (IFN-gamma and IL-2), spleen cell supernatent was evaluated by ELISA method. Fig. 24 (*in vitro*) describe the mouse spleen cells IFN-gamma release after 24 hr stimulation with non-irradiated and irradiated β-glucan in the presence of mitogen (1 ㎍/㎖). In the Con A stimulated spleen cells, 0 kGy and 10 kGy modestly enhanced the IFN-gamma release. While spleen cells stimulated with 30 kGy and 50 kGy irradiated β-glucan showed improved release of cytokines ($p < 0.05$) compared to splenocytes stimulated with 0 kGy and 10 kGy irradiated β-glucan. In correlation with IFN-gamma release we examined whether the irradiated and non-irradiated β-glucan could modulate Con A-induced Th1 associated IL-2 release. Fig. 18 (*in vitro*) describe mice splenocytes IL-2 release stimulated with non-irradiated and irradiated β-glucan for 24 h in the presence of mitogen (1 ㎍/㎖). In the Con A stimulated spleen cells IL-2 release was concomitantly augmented with the 0 kGy, 10 kGy and 30 kGy of β-glucan. Interestingly that increase was not significant whereas spleen cells stimulated with 50 kGy irradiated β-glucan showed enhanced release of cytokine ($p < 0.05$) compared to mice treated with 10, 30 kGy and non-irradiated β-glucan. The direct induction of cytokines by β-glucan from various sources is a controversial issue intensified by the diversity of β-glucan sources and methodologies involved in its preparations (Brown and Gordon, 2003). It is known that the immunomodulatory effects of β-glucans are influenced by their degree of branching, polymer length, and tertiary structure, but there is still no consensus on the basic structural requirements for biological activity. Some hypothesis prevails that IL-2 is mainly necessary during T cell development in the thymus for the maturation of a unique subset of T cells that are termed as regulatory T cells. It also plays vital role in the development of T cell immunologic memory, one of the unique characteristics of the immune system, which depends upon the expansion of the

number and function of antigen-selected T cell clones (Thornton *et al.*, 1998). Our observation reveals that *in vitro* spleen cells treated with irradiated β-glucandisplay enhanced immunostimulatory activity compared to non-irradiated β-glucan molecule. As a whole these outcomes intend a clue that immunostimulating action of irradiated β-glucan appears to be caused by events favourable for spleen cell potentiation and release of several Th1 associated cytokines to immune circulation (Trinchieri, 1995) and which would be benefical to regulate immune function especially in situations like allergy, tumor formation, intracellular pathogens ingress and endurance (Falch *et al.*, 2000; Ross *et al.*, 1999).

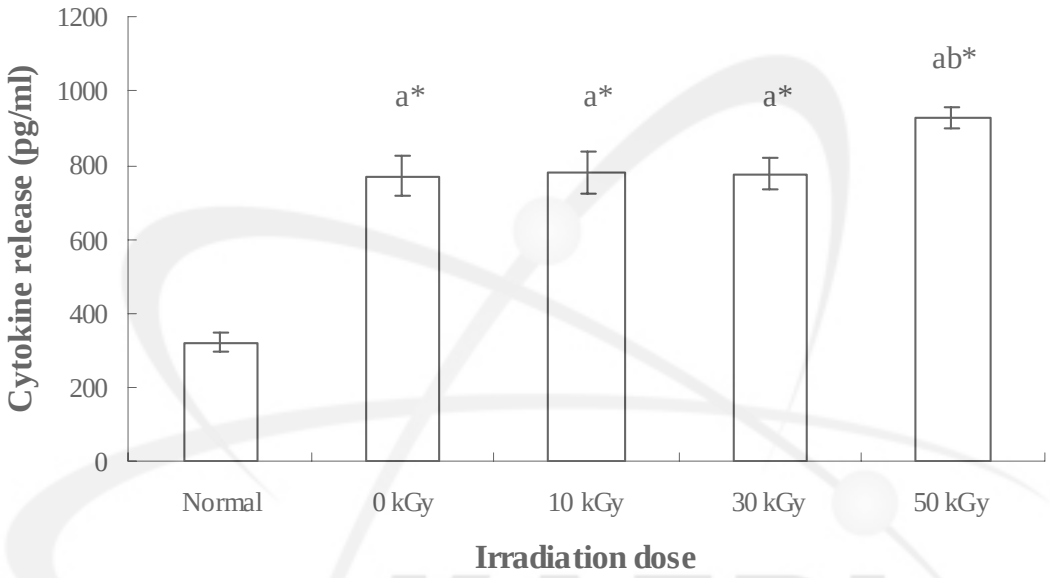

Fig. 24. Effects of non-irradiated and irradiated β-glucan and Con A (1 ㎍/㎖) on mouse spleen cell IL-2 production.

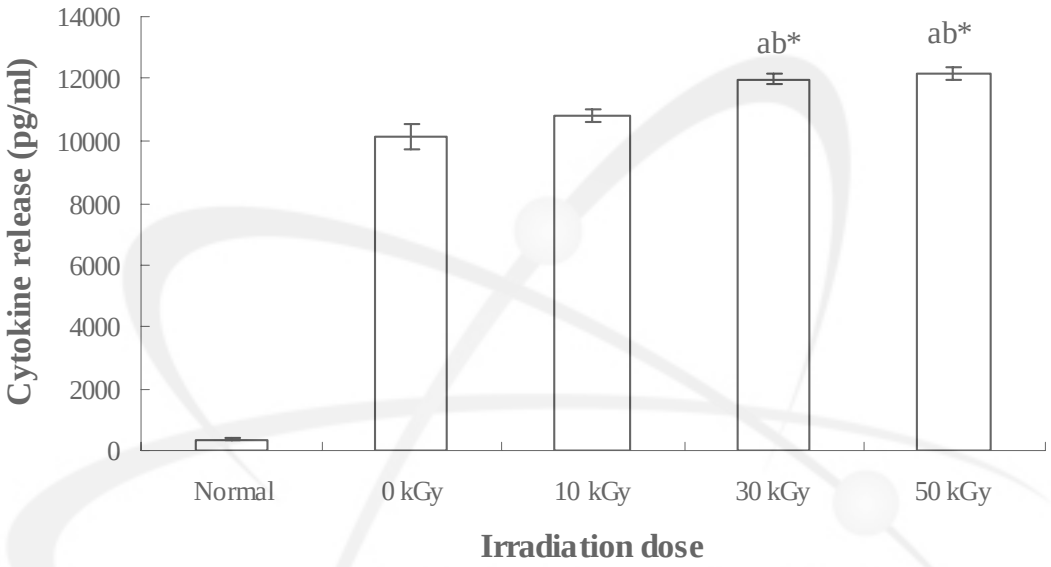

Fig. 25. Effects of non-irradiated and irradiated β-glucan and Con A (1 ㎍/㎖) on mouse spleen cell IFN-gamma production.

DPPH radical scavenging effect of gamma irradiated β-glucan

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effect of β-glucan. Antiradical effect of gamma-irradiated β-glucan is shown in Fig. 26. The DPPH radical scavenging capacity of irradiated β-glucan was relatively higher than that of 0 kGy at the same concentration. Grigorij *et al.* (2005) reported that β-glucan could completely inhibits lipid peroxidation and Göksel Sener *et al.* (2005) provided evidence for the radical scavenging activity of β-glucan by *in vivo*. We used BHT as standard reference for the study which have credible high antioxidant activity (Chatterjee *et al.*, 1999). Although radical scavenging activity of β-glucan was not significant compared to BHT, our results disclosed that gamma-irradiated β-glucan exhibts better DPPH radical scavenging capacity compared to non-irradiated β-glucan.

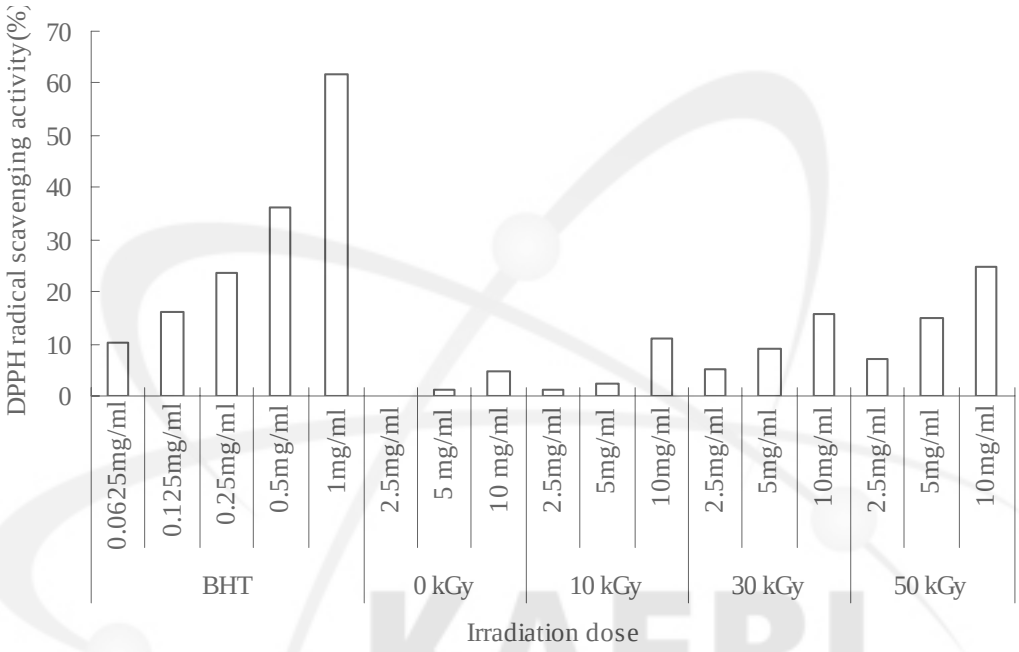

Fig. 26. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging capacity of non-irradiated and irradiated β-glucan at 10, 30, and 50 kGy. The concentration of β-glucan is arranged in 2.5, 5 and 10 ㎎/㎖. Concentration of BHT is arranged 0.0625, 0.125, 0.25, 0.5 and 1 ㎎/㎖.

Proleferative index of spleen cell by non - irradiated and irradiated β-glucan

Mice spleen cell proliferation index (Optical density) administered with non - irradiated and irradiated β-glucan are summarized in fig 27. The rate of mice spleen cell proliferation was measured by MTT assay after 7 days administration of non - irradiated and irradiated β-glucan. Spleen is an important immunological organ which contains mainly lymphocytes (Borchers *et al*., 1999). Hence, the spleen can indirectly regulate immune response under normal and pathology situation. A well-known fact exists that branched β-1,3-D-glucans originated from edible sources are their key components responsible for tumor inhibition by their immunomodulatory property (Jindrich and Mark, 1999). Our present study focused on mouse spleen cell response and cytokine release *in vivo* after consecutive adminstration of irradiated and non-irradiated β-glucan (50 ㎎/㎏ body wt) for 7 days by oral gavage. Mice treated with non-irradiated and irradiated β-glucan showed active spleen cell proliferation when compared with normal control. Mice spleen cell proliferation ($p < 0.05$) treated with 10 kGy β-glucan was 0.571 ± 0.043 (stimulatory percentage 128.5%) whereas mice administered with 30 and 50 kGy irradiated dose significantly ($p < 0.05$) enhanced the spleen cell proliferation to 0.590 ± 0.016 and 0.786 ± 0.024 (stimulatory percentages 139.3% and 180%) respectively. From this it is evident that higher the irradiation dose greater the spleen cell proliferation without any severe adverse reaction under administered dose and time period. β-glucan are well known leukocyte activators that have shown protective benefits in a variety of animal disease models, yet the mechanism of the action of these carbohydrates remains unknown. Lisa *et al*. (2006) and Jürgen *et al*. (2004) has proposed that Dectin-1, a murine type II C-type lectin-like receptor, was able to non-opsonically recognize β-1,3 and β-1,6 linked glucan rich particles and in intact yeast. It is also expressed on the surface of murine and human leukocytes. This receptor can also recognize and bind T cells and stimulate their proliferation. But few reports suggested that yeast β-glucan may have immunological activity by virtue of its interaction with gut-associated lymphoid tissue (GALT). Immune cells associated with GALT may be activated *via* their contact with yeast β-glucan in the gut and migrate to other tissues where they may exert immunomodulatory effects (www.pdrhealth.com). In the present study, we show that *in vivo* administration of irradiated β-glucan exhibits profound effect on T cell proliferation in dose dependent fashion compared to non-irradiated β-glucan.

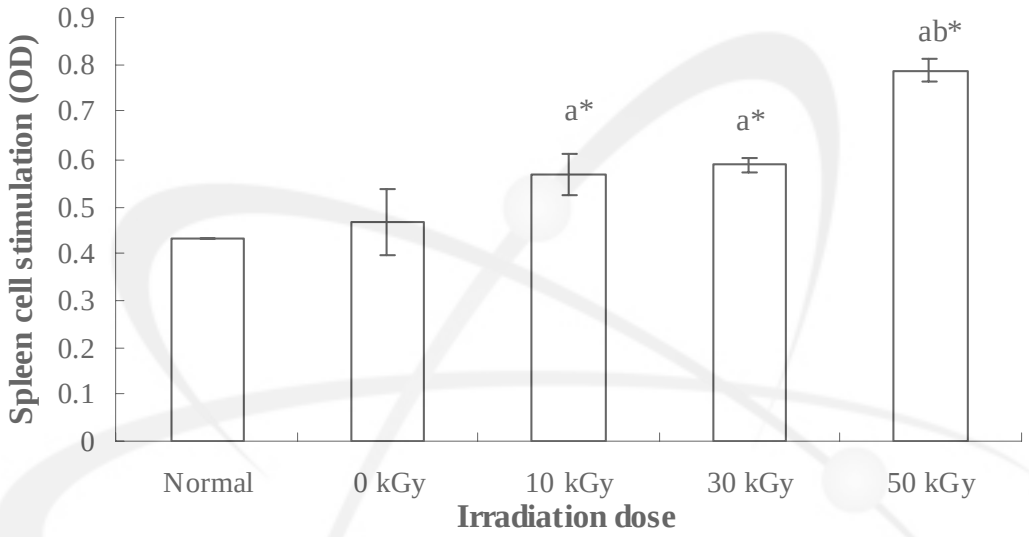

Fig. 27. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on mouse spleen cell proliferation.

Effect of irradiated β-glucan on spleen cell cytokine release

Immunomodulatory cytokines such as IFN-gamma, IL-2 and TNF-α are key in macrophage activation necessary for overwhelming different infectious agents by immunoediting mechanism (Leenen *et al.*, 1994). Protective cell-mediated immune responses in cancer condition are critically dependent on T-helper type cytokines such as interferon-gamma (IFN-gamma) where studies have also shown that IFN-gamma mediates CD4 (+) T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy (Berner *et al.*, 2007). T cells (cytotoxic and Th1) and natural killer cells secrete interferon gamma to activate macrophages that stimulate secretion of lysozyme and tumor necrosis factor (TNF), and increase the phagocytosis of antigens (Meira *et al.*, 1996). Our study was attempted to examine the immunostimulatory function of non-irradiated and irradiated β-glucan *in vivo*. The spleen cell release of Th1-associated cytokines was evaluated by ELISA method. Fig. 28 and 29 describe the level of Th1-associated cytokines IFN-gamma and IL-2 released by the mice spleen cells immunized with non-irradiated and irradiated β-glucan. The level of Th1-associated IL-2 was found to be concomitantly increased with irradiation dose of β-glucan. The mice pretreated with 0 kGy, 10 kGy and 30 kGy showed modest IL-2 release compared to control mice ($p < 0.05$) whereas the mice administered with 50 kGy showed noteworthy ($p < 0.05$) IL-2 release compared to 0 kGy.

It has been reported that lymphocytes and monocytes isolated from mice treated *in vivo* with soluble β-glucan produced a significantly different cytokine response, which was generally characterized by enhanced IFN-gamma production and suppressed production of TNF-α, suggesting that immunomodulation with soluble β-glucan might act to depress the inflammatory cytokine response (Poutsiaka *et al.*, 1993). Interestingly, Mice administered with 50 kGy irradiated β-glucan for a period of 7 days showed maximum level of cytokine release ($p < 0.05$) than normal control and mice administered with β-glucan irradiated doses (10 and 30 kGy). Whereas mice received 30 kGy showed modest increase ($p < 0.05$) in the IFN-gamma release compared to 0 kGy but statistically insignificant compared to 10 kGy. Mice administered with 10 kGy and 0 kGy showed almost similar pattern IFN-gamma release. While Adams *et al.* (1997) have reported previously that some forms of

soluble β-glucan did not induce cytokine production *in vitro* by human leukocytes or cultured murine BMC2.3 cells.

There is now a considerable body of published research detailing the biological effects of β-glucan but unfortunately the literature is inconsistent and contradictory. This has mainly been due to the use of β-glucans with different molecular weight derived from variety of sources (Gordon and Siamon, 2003). In particular the *in vivo* immunomodulatory function is far from being understood. Few recent studies started to shed light on the mechanisms behind the proinflammatory response induced by large molecular weigh β-glucan and particulate β-glucan (Sato *et al.*, 2003). Administration of intermediate or low molecular weight β-glucan has been shown to exhibit credible biological activity *in vivo* where Bohn and BeMiller (1995) reported that very short β-glucans (< 5000 ~ 10,000) generally considered inactive but their exact cellular effect is still unclear. It is generally concerned that high molecular weight β-glucan are implausible to efficiently bind to its target site in the cell at high concentration for effective immuno modulators. In our present observation we found that *in vivo* administration of irradiated β-glucan (low molecular weight) exhibit better immunostimulatory activity compared to non irradiated β-glucan. Kimura *et al.* (2007) reported that the protective role low molecular weight (LW)-β-glucan purified from *A. pullulans* 1A1 strain against food-induced allergic reactions was due to the inhibition of food antigen-specific IgE elevation through the inhibition of IFN-gamma decline induced by food antigens in the spleen cells. In addition, a patent study (Lehmann *et al.*, 2000) also reported that low molecular weight β-glucans ranging between 1 kDa ~ 30 kDa are able to display better immunomodulatory property compared to high molecular weight glucans. Although our data is obscure about the immunomodulatory mechanism of action, it emphasizes a clue for the future research that application of irradiated β-glucan (radiation dose dependency) could modulate the induction of cytokines especially in situations like sepsis, food allergy and cancer (Cristina *et al.*, 2005).

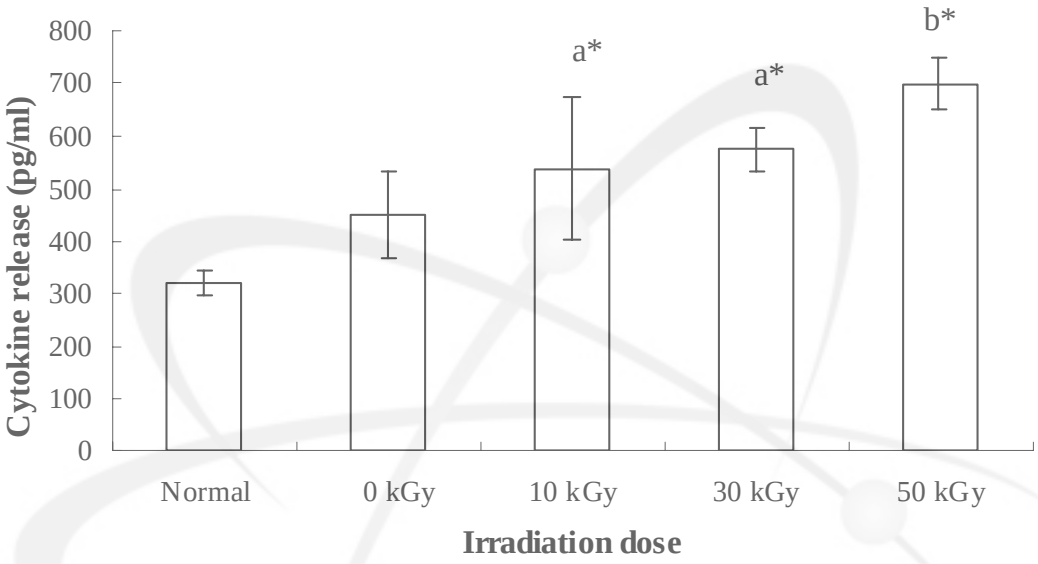

Fig. 28. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on mouse spleen cell IL-2 production.

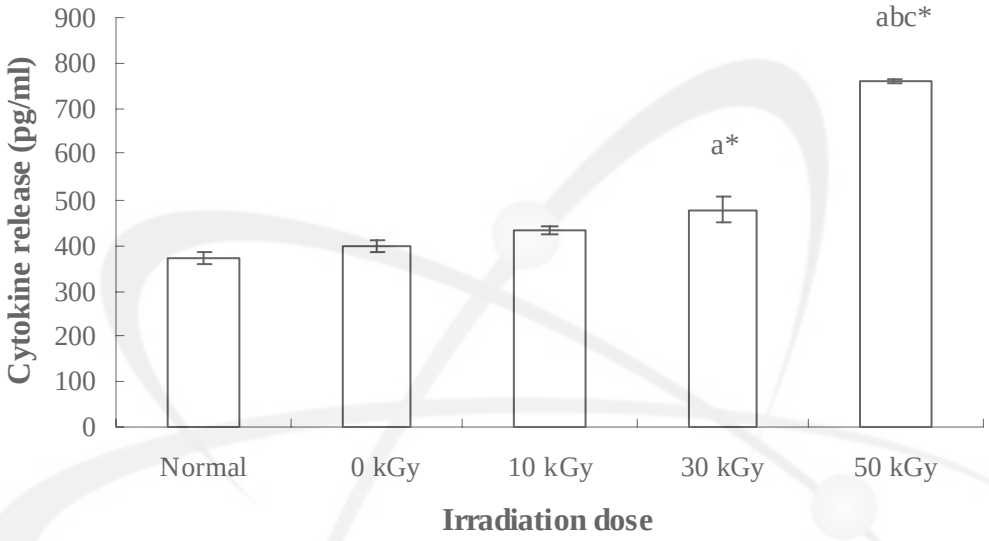

Fig. 29. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on mouse spleen cell INF-gamma production.

Body weight, spleen weight and HDL, LDL cholesterol

Mice body weight changes of irradiated and non-irradiated β-glucan (50 ㎎/㎏ body wt.) are summarized in Fig. 30. The animals treated with non-irradiated β-glucan fail to show considerable changes in the body weight compared to normal control, this result consistent with Li *et al.* (1986) observation. However the mice administered with irradiated β-glucan showed a modest increase (2%) in the body weight compared to animals administered with 0 kGy. Statistical significance was done using Wilxocon sign paired method (Normal and 0 kGy were p^{a} = 0.040 < 0.05, p^{a1} = 0.250 > 0.05, 10 kGy - p^{b} <= 0.040 > 0.05, 30 kGy - p^{c} = 0.112 > 0.05 and 50 kGy - p^{d} = 0.17 > 0.05). The effects of irradiated and non-irradiated β-glucan (50 ㎎/㎏ body wt.) on spleen weight are shown in Fig. 31. Compared to normal control, mice administered with non-irradiated β-glucan, 10, 30 kGy irradiated β-glucan moderately altered the spleen weight, but 50 kGy irradiated β-glucan showed noteworthy elevation in the spleen weight. It has previously been reported that peritoneal injection of glucan derivatives increased mice (ICR/HSD) spleen weight, and induced hyperplasia of splenic follicles and appearance of many multinucleate giant cells (Williams *et al.*, 1988; Toshie *et al.*, 2005) which are contradictory with the our results. Possible explanations for these differences are related to the dose and mode of β-glucan adminstration (50 ㎎/㎏ b.wt intragastric intubation for 7 days). However, further studies are necessary to investigate this issue.

Beside its immunomodulatory property β-glucan has also received much attention because of its potential to reduce low density lipoprotein (LDL) cholesterol. The effect of non-irradiated and irradiated β-glucan on BALB/C mice blood cholesterol (HDL & LDL) presented in Fig. 32, 33. The mice administered with 10 kGy, 30 kGy and 50 kGy irradiated β-glucan showed relatively low level of LDL than non irradiated β-glucan. Mice administered with 10 kGy irradiated showed (p < 0.05) significance compared to 0 kGy. But mice administered with 30 kGy and 50 kGy showed low level of LDL significance was not high. LDL cholesterol lowering effect has become an important focus with a variety of experimental, genetic and epidemiological studies linking high levels of low-density lipoprotein (LDL) cholesterol to an increased risk of heart disease. Ingestion of soluble β-glucan has been shown to improve the pattern of lipids, in humans and experimental animals,

with elevated serum cholesterol. In clinical studies the reversal of hypercholesterolemia has been demonstrated with dietary supplementation of oats or oat bran and purified β-glucans from yeast. It is thought that β-glucan reduces cholesterol levels by increasing excretion of cholesterol from the digestive tract. Fig. 33 show the effect of irradiated β-glucan on HDL cholesterol, but a same or very mild HDL increase were observed in mice administered with 10 kGy, 30 kGy and 50 kGy irradiated β-glucan. In general, the mechanism behind LDL cholesterol lowering activity of β-glucan is not clear, but the researchers proposed that β-glucan bound to bile acids, thereby preventing reabsorption in the intestine and increasing excretion (http://www.creanutrition-sof.com/mechanism_of_action.html). Even though there was a slender modulation in the levels of cholesterol we ascertain that the increase in mice body weight, spleen weight and cholestrol were of no correlation. However mice treated with irradiated β-glucan showed low level of LDL with improved body weight. From this results it is tempting to anticipate that animals may utilized β-glucan of low molecular weight and viscosity which could be the reason for both cholesterol lowering effect and immunomodulatory property consistent with the patent study where β-glucan with molecular weight roughly less than 30,000 Daltons possess improved optimum absorption with beneficial biological properties. Thus we propose that using irradiated β-glucan may improve the spleen cell host recognition to deter antigens with improved immunomodulatory property.

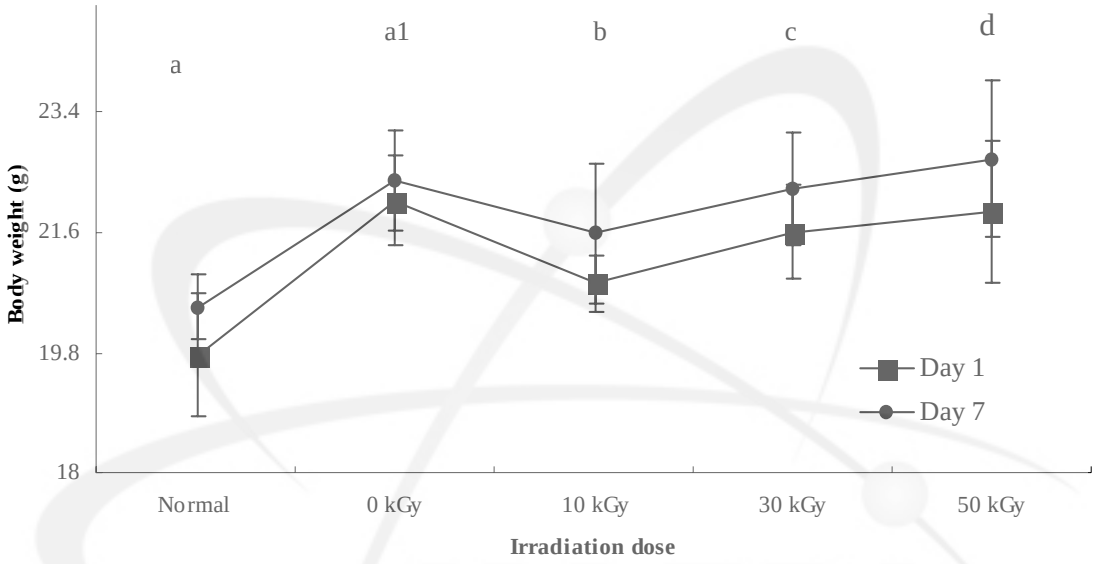

Fig. 30. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on body weight changes. Results are expressed as the mean ± S.D of 6 mice. Weight changes compared between day 1 and 7 of each group.

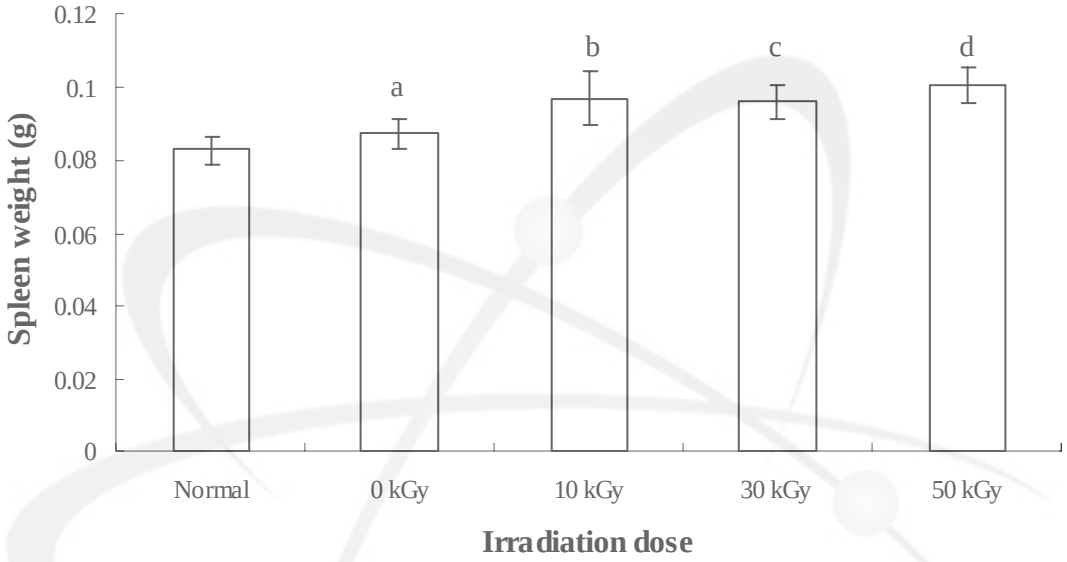

Fig. 31. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on spleen weight changes.

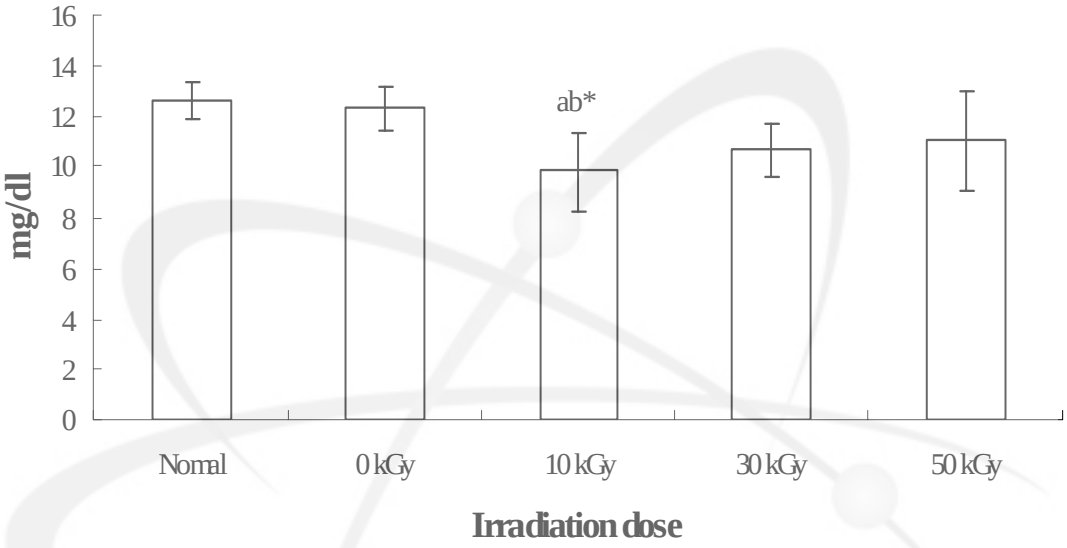

Fig. 32. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on mouse serum LDL cholesterol.

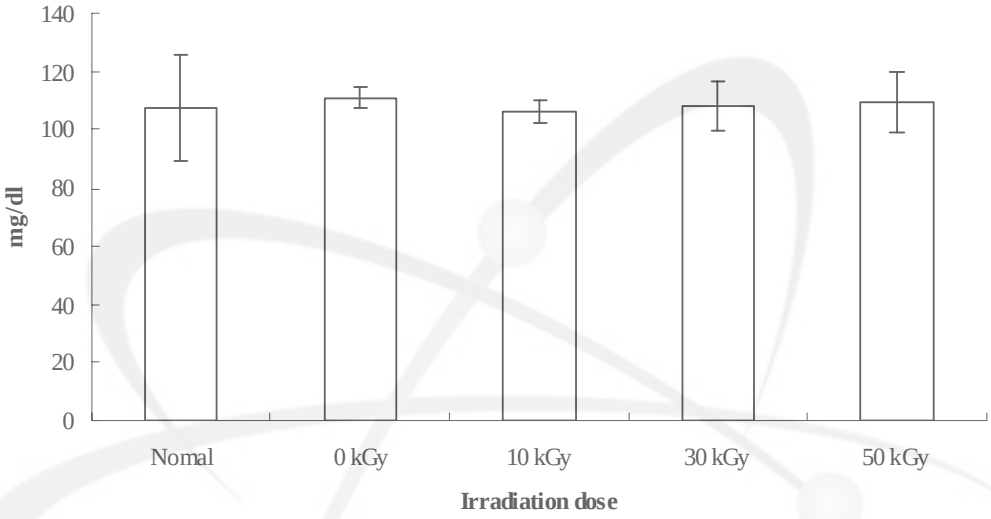

Fig. 33. Effects of β-glucan non-irradiated and irradiated (50 ㎎/㎏ b.wt/day for 7 days, intragastric intubation) on mouse serum HDL cholesterol.

제3절 차가버섯 폴리페놀의 색상 개선 및 항산화활성 증진기술 개발

1. INTRODUCTION

Recently, mushroom used for a medicine has become attractive as a functional food. The Chaga mushroom (*Inonotus obliquus*) is a black parasitic fungus that grows on living trunks of the mature birch tree in cold northern climates. Many researches have reported that Chaga mushroom, possesses various biological properties such as hypoglycemic, anti-inflammatory, anti-viral, anti-fungal, antioxidant activity, immuno-modulatory, and anti-tumor properties (Kahlos, 1994; Kahlos, 1996; Nakajima *et al*., 2007; Park *et al*., 2005; Shin *et al*., 2000; Cui *et al*., 2005; Lee *et al*., 2007). In spite of all these beneficial effects, Chaga mushroom has been consumed mostly for drinking because of its deep dark color and off-flavor, which makes it very difficult to apply the proper amount in cosmetics, medicine, or foods industries.

Gamma irradiation is also well known as the effective method for eliminating pathogenic and spoilage microorganisms without compromising the nutritional properties and sensory quality of foods, and its use has gradually been increased worldwide (WHO 1999). Additionally, irradiation technology has been used as a tool for improving the color properties of natural extracts such as green tea (Jo, 2003), *Curcuma aromatica* (Kim *et al*., 2006), persimmon and licorice root (Jo *et al*., 2003) without any adverse change in their physiological activities. Furthermore, many studies have shown that irradiation affected the antioxidant properties of various foods and natural extracts. However, there were not many studies to evaluate gamma irradiation effects on the color and antioxidant activity of Chaga mushroom extract (CME). Therefore, the present study examined the effects of gamma irradiation in obtaining a light-colored CME and antioxidant activity.

2. MATERIALS AND METHODS

Sample preparation

Dried Siberian Chaga mushroom was purchased from Korea Tabacco & Ginseng Corporation, Korea. One hundred grams of dried Chaga mushroom were crushed in grinder(FM-681, Hanil, Seoul, Republic of Korea) for 2 min and extracted with 1 L of deionized water in a water bath (E-320, LAUDA, Germany) at 70℃ for 4 h. The CME was then filtered using Whatman No.4 filter paper (Whatman International Ltd., Springfield Mill, Kent, England) andlyophilized into powder.

Irradiation

The lyophilized CME powder was solubilized in deionized water (10 ㎎/㎖) and transferred into a 50 ㎖ capped glass tube. This solution was then irradiated at 0, 3, 5, 7, and 10 kGy by a cobalt-60 gamma-irradiator (IR-221, Nordion International Ltd., Ontario, Canada) equipped with a 11.1 PBq source strength at 10 ± 0.5℃ and operated at a dose rate of 10 kGy/h. Dosimetry was performed with 5-mm-diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). After irradiation the irradiated samples were stored at 4℃.

Color measurement

Samples were transferred into a glass cell (CM A-98, 10 mm in width), and lightness (L), yellowness (a), and redness (b) were measured with the Color Difference Meter (Spectrophotometer CM-3500d, Minolta Co., Ltd. Osaka, Japan) in triplicate. The Hunter color L-, a-, and b-values were reported through the computerized system using Spectra Magic software (version 2.11, Minolta Cyberchrom Inc. Osaka, Japan). The instrument was calibrated to standard black and white tiles before analysis.

DPPH radical scavenging activity

One ㎖ portions of diluted CME samples were mixed with 1 ㎖ of 0.2 mM 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Co., St. Louis, MO, USA) dissolved in methanol to obtain final concentrations of 3.91, 7.81, 15.63, and 31.25 ㎍/㎖ of CME. The mixture was vortexed and left to stand for 30 min at room temperature, and

absorbance was measured at 517 nm with a spectrophotometer (UV-1601PC, Shimadzu, Japan). Blank control and blind control were a mixture of CME and pure methanol and a mixture of DPPH and distilled water, respectively. Following equation was used for the calculation of the DPPH scavenging activity

$$\text{Scavenging activity } (\%) = (1- (A^{sample}_{517} - A^{blank}_{517})/(A^{blind}_{517})) \times 100$$

Where the scavenging activity refers to the free-radical scavenging percentages, A^{sample}_{517} refers to the absorbance of a sample plus DPPH solution, and A^{blank}_{517} refers to the absorbance of the sample as a blank control, and A^{blind}_{517} refers to the absorbance of the blind control.

In order to find whether irradiation influenced levels of polyphenolic compounds in CME, Polyclar® (PVPP (Polyviny l-polypyrrolidone), ISP, Wayne, NJ. USA) which is able to absorb polyphenolic compounds was added to CME (10 ㎎/㎖) to remove polyphenolic compounds from the extract according to the manufacturer's instructions (Sawabe *et al.*, 1998), and anti-oxidant activity of CME was then determined as described above.

Ferric reducing/antioxidant power (FRAP) assay

A FRAP reagent containing 2.5 ㎖ of a 10 mM TPTZ solution (2,4,6-tripyridy-s-triazine, Sigma) in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ㎖ of 0.3 M acetate buffer (pH 3.6) was prepared and warmed at 37℃. Thirty ㎕ of irradiated CME samples (2 ㎎/㎖) were mixed with 0.09 ㎖ distilled water and 0.9 ㎖ of FRAP reagent. Then mixture of CME and distilled water was used as a sample blank. The absorbance of the sample was then measured at 593 nm by a spectrophotometer after incubation at 37℃ for 10 min. The Fe (II) solution (20-2000 ㎛M) was used for measuring concentration of Fe (II) in CME samples.

Radical scavenging activity by ultra weak chemiluminescence

Superoxide and hydroxyl radical scavenging ability was estimated by ultraweak chemiluminescence assay (UWLA) according to modified method (Tasi *et*

al., 2003; Tasi *et al*., 2004). To determine IC_{50} (a CME concentration reducing radical by 50%) of the non-irradiated and irradiated CME, UWLA kits (BJH Inc, Taipei) was used according to the manufacturer's instructions. The ultraweak photon produced after UWLA kit application was measured using an ultraweak chemiluminescence (CL) analyzer (Jye Horn Co, Taipei, Taiwan) with a high sensitivity (^{14}C-light source generating 10,000 photon counts/s at a voltage range between 860 and 867 V). The reproducibility of the ^{14}C-light source was < 1.0%. Luminescence intensity was monitored at the wavelength range from 260 to 750 nm. For the determination of the hydroxyl radical scavenging ability, 12 min after the initiation of the CL reaction generated by UWLA kit specialized for hydroxyl radical in the CL analyzer (CL versus time relationship can be visualized on the screen of the data processing unit), 2 ㎕ of CME (0.5 ㎎/㎖) to the UWLA kit was added at different time intervals to plot radical inhibition curves of CME.

For the determination of the superoxide radical scavenging ability, 12 min after the initiation of the CL reaction generated by UWLA kit specialized for superoxide radical in the CL analyzer, 2 ㎕ of CME (0.1 ㎎/㎖) was added to the UWLA kit. As a result, varying degrees of sudden drops of CL counts could be observed lower CL counts indicated higher hydroxyl and superoxide radical scavenging abilities. IC50 was then determined (Tasi *et al*., 2003; Tasi *et al*., 2004).

Total levels of phenolic compounds

Each CME sample (0.9 ㎖) at a concentration of 2 ㎎/㎖ was mixed with 0.1 ㎖ of ascorbic oxidase (50 units/㎖) followed by incubation at 23℃ for 90 min to remove the ascorbic acid. The L-ascorbic acid-free CME (0.1 ㎖) was then mixed with 0.2 ㎖ of Folin-Ciocalteau reagent (SigmaChemical Co., St. Louis, MO, USA) and incubated at 23℃ for 1 min. Following the incubation, 3 ㎖ of 5% Na_2CO_3 was added and incubated at 23℃ for 2 h. Absorbance of this mixture was then measured at 765 nm by spectrophotometer. To calculate total phenolic compound levels in CME samples the absorbance values were concentrated to concentration (㎍/㎖) using a standard curve prepared with gallic acid was expressed as gallic acid (Sigma Chemical Co., St. Louis, MO, USA).

Rancimat test

In other to measure the antioxidant potency of the irradiated CME samples, the CME sample (0.9 g) and Tween 80 (0.1 g) were added to 3 g of corn oil, and inhibitory activity of CME against lipid peroxidation was measured by Metrohm Rancimat (Model 743, Herisan, Switzerland). The Rancimat was operated at 120℃, and a dry air flow rate was 20 L/h. The time (in h) taken to reach a specific conductivity value, corresponding to the flex point of the peroxidation curve, was considered as the induction period(*IP*). The effect of CME samples on retarding the corn oil oxidation, interpreted as the protection factor (*Pf*), was calculated according to the following expression:

$$Pf = IP_{sample} / IP_{control}$$

Where, the IP_{sample} refer to the elapsed time of CME samples to reach a specific conductivity and the $IP_{control}$ refer to the elapsed time of control (corn oil without CME) to reach the specific conductivity. The higher the *Pf* is, the higher the antioxidant potency of the compounds. All tests were performed in triplicate.

Statistical analysis

The data were analyzed by a Statistical Package for the Social Science (SPSS Inc., 10.0, 2000). Differences among the mean values were examined by student's two tails *t*-test (Fig. 34, 35) and Duncan's multiple comparison tests (Table 5, 6, 7, 8, 9) with significant level of $p < 0.05$.

3. RESULTS

Color

The changes of the Hunter color values of CME after gamma irradiation are shown in Table 5. The lightness (*L*-value) and yellowness (*b*-value) were significantly increased ($p < 0.05$) with an increase of the irradiation dose, but the

redness (a-value) was significantly decreased ($p < 0.05$) as the irradiation dose increased. This result showed that the dark color of CME became bright by gamma irradiation.

Table 5. Hunter's color L (lightness), a (redness), b (yellowness) values (mean ± standard deviation) of the CME (n=3) after gamma irradiation

Irradiation dose (kGy)	L-value	a-value	b-value
0	56.7 ± 0.008^{e}	28.5 ± 0.002^{a}	68.1 ± 0.048^{e}
3	58.6 ± 0.006^{d}	28.3 ± 0.007^{b}	76.1 ± 0.063^{d}
5	59.7 ± 0.001^{c}	27.5 ± 0.004^{c}	76.8 ± 0.040^{c}
7	60.6 ± 0.0003^{b}	27.0 ± 0.007^{d}	79.7 ± 0.03^{b}
10	62.5 ± 0.01^{a}	25.0 ± 0.0002^{e}	81.0 ± 0.031^{a}

$^{a, b, c, d, e}$Means values with different superscript letters within a same column are significantly different at $p < 0.05$.

DPPH radical scavenging activity

The changes of DPPH radical scavenging activity of CME after gamma irradiation are shown in Table 6. The inhibitory concentration (IC_{50}) of CME for DPPH radical scavenging was decreased with an increase of the irradiation dose. The IC_{50} values of non-irradiated and irradiated CME at 3, 5, 7 and 10 kGy were 21.9 ± 0.05, 22.0 ± 0.38, 18.2 ± 0.27, 18.4 ± 0.95 and 16.8 ± 0.61 ㎍/㎖, respectively. To find a reason for the increase in antioxidant activity of CME by gamma irradiation, the CME without polyphenolic compounds was prepared using polyclar® and irradiated. However, when the polyphenols were removed from CME, DPPH radical scavenging activities were not significantly changed ($p > 0.05$) by irradiation (Fig. 34). This result indicated that irradiation changed polyphenolic compounds which resulted in the increase of the antioxidant activity.

Table 6. DPPH Radical scavenging activity (mean ± standard deviation) of the CME (n=3) after gamma irradiation

Irradiation dose (kGy)	IC_{50} values(㎍/㎖)
0	21.9 ± 0.05[a]
3	22.0 ± 0.38[a]
5	18.2 ± 0.27[b]
7	18.4 ± 0.95[b]
10	16.8 ± 0.61[c]

[a, b, c]Means values with different superscript letters within a same column are significantly different at $p < 0.05$.

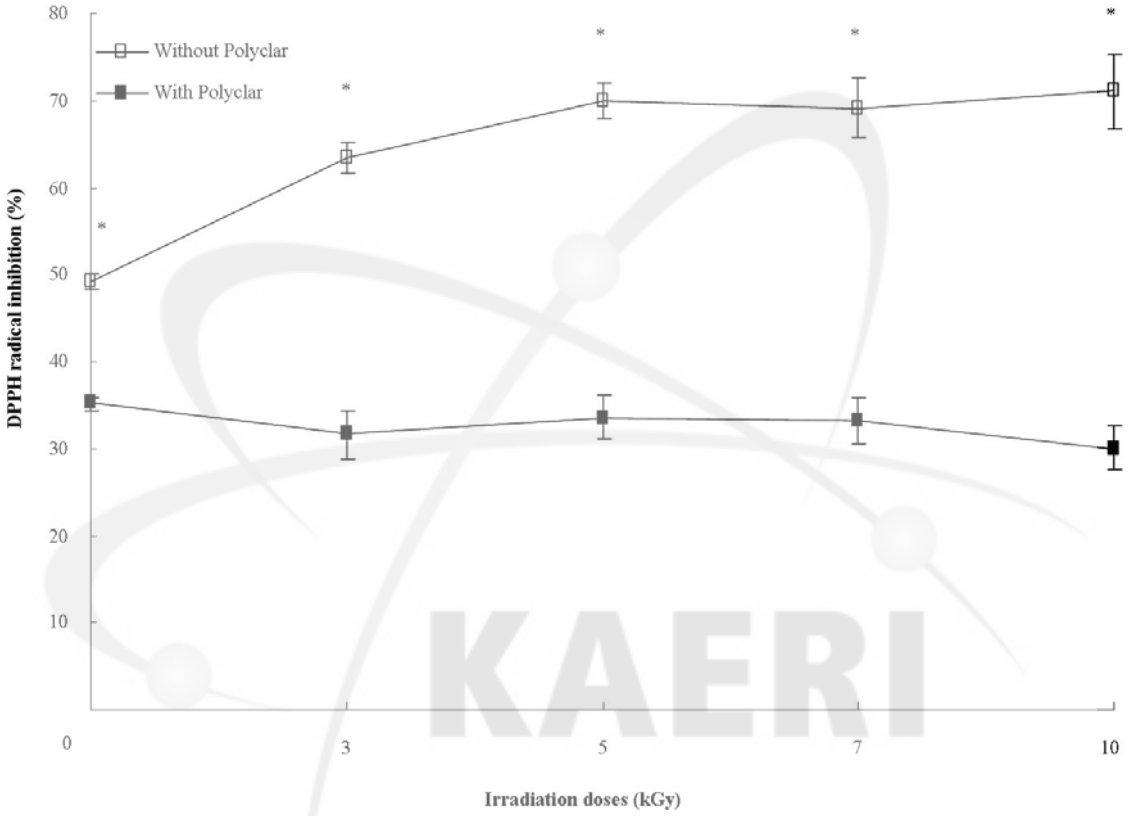

Fig. 34. DPPH radical scavenging activity of gamma irradiated CME at dose 25 ㎍/㎖ without and with polyclar®. $^{*}p < 0.05$ compared to polyclar group.

FRAP value

Table 7 shows the changes of FRAP value of irradiated CME. The FRAP value of CME was also significantly increased ($p < 0.05$) as the irradiation dose increased. The FRAP values of non-irradiated and irradiated CME at 3, 5, 7, and 10 kGy were 1678 ± 25.87, 1761 ± 9.30, 1936 ± 18.68, 2025 ± 11.83, and 2169 ± 104.09 μM/mℓ, respectively.

Table 7. FRAP values (mean ± standard deviation) of the CME (n=3) after gamma irradiation

Irradiation dose (kGy)	FRAP value (Fe^{2+} μM of sample)
0	1678 ± 25.87^{c}
3	1761 ± 9.30^{c}
5	1936 ± 18.68^{b}
7	2025 ± 11.83^{ab}
10	2169 ± 104.09^{a}

$^{a, b, c}$Means with different superscript letters within a same column are significantly different at $p < 0.05$.

Superoxide and hydroxyl radical scavenging capacities

Scavenging capacities of CME samples against superoxide and hydroxyl radicals are shown in Table 8. IC_{50} against superoxide radical was found to be decreased ($p < 0.05$) significantly as the irradiation dose increased from 3 to 10 kGy. Moreover, irradiation improved both hydroxyl and superoxide radical scavenging activity of CME, but more increase was observed in superoxide radical scavenging activity than in hydroxyl radical scavenging activity.

Table 8. Superoxide and hydroxyl radical scavenging activities (mean ± standard deviation) the CME (n=3) after gamma irradiation

Irradiation dose (kGy)	Radical scavenging activity IC_{50} (㎍/㎖)	
	Superoxide radical	Hydroxyl radical
0	3.19 ± 0.06[a]	1.72 ± 0.03[a]
3	2.38 ± 0.05[b]	1.48 ± 0.02[b]
5	2.12 ± 0.05[b]	1.48 ± 0.01[b]
7	0.85 ± 0.04[c]	1.41 ± 0.05[b]
10	0.45 ± 0.07[d]	1.38 ± 0.05[b]

[a, b, c, d]Means with different superscript letters within a same column are significantly different at $p < 0.05$.

Total phenolic compound concentrations

Total phenolic compound concentrations of the CME irradiated at 0, 3, 5, 7, and 10 kGy were 88.7 ± 0.22, 92.3 ± 0.27, 94.6 ± 0.34, 96.1 ± 0.15, and 98.0 ± 0.28 ㎍/㎖, respectively (Table 9). This result suggested that gamma irradiation increased total phenolic compound concentrationsof CME. The maximum total phenolic compound concentrations of irradiated CME were observed in 10 kGy at a concentration of 2 ㎎/㎖ CME.

Table 9. Total phenolic compound concentrations (mean ± standard deviation) of the CME (n=3) after gamma irradiation

Irradiation dose (kGy)	Total Phenolic compound concentrations (㎍/㎖)
0	88.7 ± 0.22[e]
3	92.3 ± 0.27[d]
5	94.6 ± 0.34[c]
7	96.1 ± 0.15[b]
10	98.0 ± 0.28[a]

[a, b, c, d, e]Means with different superscript letters within a same column are significantly different at $p < 0.05$.

Rancimat test

Each irradiated and non-irradiated CME samples were added to corn oil to obtain a final concentration of 1 ㎎/㎖, and Fig. 35 shows the inhibition of lipid peroxidation of CME. A significant increase ($p < 0.05$) of *Pf* value was observed between the control and non-irradiated sample (0 kGy), but no differences ($p > 0.05$) of *Pf* values were observed between non-irradiated and irradiated samples regardless of irradiation dose.

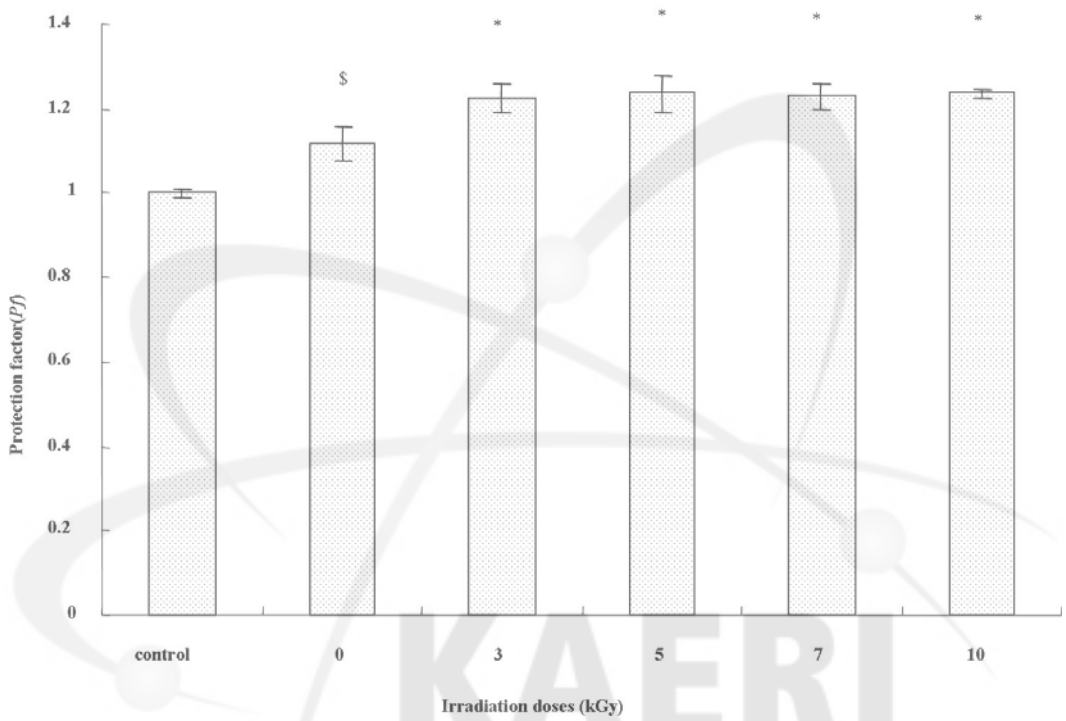

Fig. 35. Antioxidant effect (*Pf*) of gamma irradiated CME, tested by Rancimat method. $^{\$}p < 0.05$ compared with control, $^{*}p < 0.05$ compared with 0 kGy.

4. DISCUSSION

Irradiation has direct and indirect effects. In a case of direct effect, ionized particles or rays directly affect target food components, but in other case, irradiation exerts have its effect through a radiolysis of water, which results in the production of radicals such as hydrated electrons, hydroxyl radicals, and hydrogen atoms (Stewart, 2001). It appears that irradiation could change the physicochemical and structural properties of various food components. In this study, there was a significant improvement in the color of irradiated CME. This is consistent with the previous studies which showed that the dark color of natural extracts from foods or medicinal herbs became bright by gamma irradiation (Jo *et al*., 2003; Kim *et al*., 2006; Jo *et al*., 2004). One of the possible reasons for the discoloration of CME by irradiation might be due to the oxidation of color compounds such as carotenoids. Thus, irradiation technology could be a potential mean for making CME brighter and more commercially useful ingredient for food and cosmetic industries.

Phenolic compounds are commonly found in mushrooms, and they have been reported to have multiple biological effects, especially an antioxidant activity (Huang and Mau, 2006). The antioxidant activity of polyphenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals quenching singlet and triplet oxygen, or decomposing peroxides. It is well known that CME possesses the strongest antioxidant activity compared with those of among other medicinal mushrooms (*Agaricus blazei*, *Ganoderma lucidum*,*and Phellinus linteus*) examined in terms of both superoxide and hydroxyl radical scavenging activities (Nakajima *et al*., 2007; Valentao *et al*., 2002). In the present investigation, the effects of gamma irradiation on the antioxidant activity of CME were evaluated by the analysis of DPPH, hydroxyl and superoxide radical scavenging activity, reducing power and lipid peroxidation. The results found in our study, the irradiated CME was found to have an increase in antioxidant activity compared to those of non-irradiated CME. It has also been reported that the radical scavenging ability of green tea leaf extract (Jo *et al*., 2003), phytic acid (Ahn *et al*., 2003), and soybean (Variyar *et al*., 2004) was significantly increased by irradiation.

Kahlos *et al*. (1987) suggested that if multimeric compounds were degraded to monomeric compounds, their antioxidant activity could be enhanced. Adamo *et al*.

(2004) found that the polyphenols of truffles were degraded by irradiation, giving rise to an increase of soluble phenols. Similarly, Variyar *et al.* (2004) found that in soybean irradiated at 0.5 to 5 kGy, the glycosidic conjugatescontent related to phenolic compounds was decreased when radiation dose increased. Furthermore, Harrison and Were (2007) reported that the increased phenolic content in gamma-irradiated almond skin extract could be attributed to the release of phenolic compounds from glycosidic components and the degradation of larger phenolic compounds into smaller compounds by gamma irradiation. In our study, when polyphenols were removed from CME, DPPH radical scavenging activities of CME were not significantly changed by irradiation. Taken together, a possible reason for the increase in the antioxidant activity of CME by gamma irradiation might be due to a change of polyphenol compounds from polymeric to monomeric phenolic compounds.

In conclusion, gamma irradiation improved the color of CME and increased its antioxidant activities, such as ferric reducing/antioxidant power (FRAP) and scavenging activities against DPPH, superoxide and hydroxyl radicals, and inhibition of the lipid peroxidation. However, further studies are needed to elucidate mechanisms of structural change and identification of the phenolic compounds formed during gamma radiation processing of CME.

제4절 방사선 생물영향 평가 및 검출기법 개발 : 방사선 및 염화수은(Ⅱ)이 어류간암세포 사멸에 미치는 상승작용 평가

1. 서 론

모든 생물체는 생활환경에 존재하는 다양한 화학물질과 물리적, 화학적, 사회적 요인의 영향을 받고 있으며, 특히 수서생물은 주위 환경으로부터 직접적인 영향을 받는다고 볼 수 있다. 수서생물이 노출 가능한 요인으로는 수중으로 흘러들어온 각종 중금속, 농약, 인공 및 자연 방사선 등 화학물질과 수온, pH, 빛, 유속 등의 물리적 요인, 각종 요인들 간의 화학적 반응, 각 생물 간의 생태적 상호작용 등에 의해 영향을 받게 된다. 그러나 화학물질에 생물이 노출될 경우, 타 요인들에 비해 체내에 미치는 영향 즉, 손상의 정도가 크기 때문에 생물, 환경, 생태계 등 관련 분야에서는 더욱 활발한 연구가 진행되고 있다. 한편, 두 가지 이상의 요인들이 복합적으로 작용할 경우, 생물체에 유발되는 영향은 복잡하고 다양하여 평가나 사전 예측이 매우 어렵기 때문에 각 물질의 동시노출에 따른 생물체내의 상승, 가산, 길항작용 등 복합작용 규명의 필요성이 제기되고 있다. 다중 요인의 복합작용을 연구하기 위해서는 자극에 보다 민감하게 반응하는 생물을 이용하여 이들의 생체내 손상 정도를 평가 및 예측하는 연구가 선행되어야 함은 물론, 효과적인 생물체내의 검정 기술의 확립이 필요하다.

화학물질 중 하나인 중금속은 노출되는 생물종이 다양하고 농도에 대한 영향이 뚜렷하여 많은 연구가 진행되었으며, 중금속의 이용이 증대됨에 따라 이에 대한 안전성 연구는 생물학적 영향을 근거로 하여 범위가 점차 확대되고 있다. 중금속 중에서도 수은화합물은 각종 환경매체를 통해 생물에 직접 노출될 가능성이 있으며, 수은화합물 중에서도 염화수은(Ⅱ)은 주로 신경계, 신장, 간 등에 손상을 유발하는 것으로 보고되었다(Emanuelli *et al.*, 1996). 염화수은(Ⅱ)에 의해 유발된 간독성에 대해서는 간세포를 이용하여 형태학적 변화를 관찰한 연구를 비롯하여 간독성과 미토콘드리아 손상의 연관성이 연구된 바 있다(Muller, 1986; Nieminen *et al.*, 1990; Sokol *et al.*, 1993).

중금속과 더불어, 방사선의 이용도 점차 증가하면서 방사선의 자극에 대한 생물의 반응을 연구하는 방사선생물학을 포함하여 방사선과 관련한 방사선물리학, 방사선화학 등 기초과학 분야의 발전을 위해 많은 연구가 진행되고 있다. 그러나 국내, 외 대부분의 실험은 이온화 방사선의 단독노출에 의한 영향 연구에 초점이 맞추어져 있어, 방사선과 타 화학물질의 동시노출에 따른 생물체내의 상승, 가산, 길항작용 등의 복합작용 규명 연구의 필요성이 제기되고 있다. 다양한 생물을 대상으로 하여 이온화 방사선과 타 화학물질

간의 복합작용을 연구한다면 더욱 효과적인 결과를 얻을 수 있다. 또한, 사람을 포함한 포유류가 중심이 된 연구에서 벗어나 실제 환경에서 노출 가능한 환경생물종의 연구를 통해 각 생물종의 비교는 물론, 생태계에 미치는 영향 예측이 가능하다.

국내·외 대부분의 연구는 세포 수준에서의 수은 또는 방사선의 단독 처리에 의한 영향을 관찰하는데 초점이 맞춰져 있으며, 실제 복합작용 연구가 보고되었다 하더라도 사람세포에 제한적으로 수행되어 왔다. 복합작용에 관한 연구사례로는 자궁경부암 세포를 대상으로 수은과 이온화 방사선을 동시 처리 후, DNA 손상 및 수복 연구가 보고된 바 있다(Woo *et al*., 2006). 따라서, 본 연구에서는 수환경 오염으로부터 직접 노출되는 생물인 어류의 세포를 대상으로 이온화 방사선과 수은에 동시에 노출되었을 경우, 그 손상을 생존율 변화를 통해 관찰하고자 하였다.

2. 재료 및 방법

세포배양

본 연구에서 사용된 어류간암세포, PLHC-1 (Poeciliopsis lucida hepatocellular carcinoma) 세포주는 topminnow의 암컷 성체의 간암세포에서 분주한 것으로, ATCC® (#CRL-2406, USA)사에서 분양받은 후, 배양하여 본 시험에 사용하였다. 30℃에서, 5% CO2와 습도가 조절되는 배양기에 배양하며, 5% FBS (fetal bovine serum)이 포함된 Eagle's Minimum Essential Medium (EMEM)의 배지를 사용하여 배양하였다. 배지는 2 ~ 3일마다 교환해주며, 계대배양은 3 ~ 5일에 한 번 1:4 비율로 하였다. 배양된 세포는 0.05% Trypsin EDTA 용액을 처리하여 분리하였으며, 만들어진 세포현탁액은 Hemocytometer으로 계수하여 세포수가 2×10^5 cells/㎖이 되도록 조절한 후, 시험을 수행하였다.

방사선 및 염화수은 (Ⅱ) 처리

PLHC-1 세포를 96-well plate에 분주하여 24시간 동안 배양한 후, 수은 및 방사선 처리를 실시하였다. 실험은 수은 및 방사선 단독 처리군과 수은과 방사선 복합처리군으로 나누어 수행하였으며, 염화수은(Ⅱ)처리 농도는 1 ~ 500 uM 사이에서 구배하여 24, 48시간 동안 처리하였다. 이온화 방사선은 한국원자력연구원의 ^{60}Co γ-ray를 이용하여 총선량이 500 Gy 이하가 되도록 조절하여 조사하였다. 본 연구에서는 방사선과 수은 복합처리군의 경우 수은 처리 후 방사선 조사 방법으로 처리하였으며, 각각의 처리 후, 단독처리군과 동일한 시간의 배양시간을 거친 후 생존율을 측정하였다.

각 처리군의 세포 생존율 측정

세포의 생존율은 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay 방법을 이용하여 측정하였다. 이 방법은 생존 세포의 탈수소 효소작용을 이용하여 처리물질에 의해 세포가 사멸 또는 증식 억제되는 정도를 결정하는 실험법으로 다양한 세포주에 적용이 가능하고 많은 시료를 간단, 신속하고 객관성 높게 판독할 수 있어 세포독성 측정 시 널리 사용되는 기법이다 (Mossmann, 1983). 수은과 방사선 처리가 끝나면 배양액 100 ㎕ 제거 및 MTT 용액 10 ㎕ 첨가 후 4시간동안 배양기에서 반응시킨다. 형성된 formazan crystal을 용해시킨 후, ELISA reader (Multiskan® EX,

Forma Scientific, Inc)를 사용하여 570 nm와 690 nm의 파장으로 흡광도를 측정하여 세포의 생존율을 계측하였다.

통계학적 분석

대조군과 실험군과의 유의성 검정은 Student's t-test로 비교하였으며, p가 0.05이하인 것만 유의한 것으로 하였다.

3. 결 과

방사선 단독 처리된 세포의 생존율

방사선이 세포의 생존에 미치는 영향을 알아보기 위하여 방사선에 노출시키고 24시간, 48시간의 배양시간을 거친 후 MTT assay를 실시하였다. 방사선 총선량 0 ~ 500 Gy 의 범위에서 처리된 각 세포의 생존은 대조군 생존율을 100%로 환산하여 비교하였다. 그 결과, 노출 시간에 상관없이 방사선 총선량 증가에 따라 세포의 생존은 유의하게 감소하였다(Fig. 36). 처리 선량 10 ~ 100 Gy의 경우 대조군과 비교하여 처리 후 24시간의 결과 값에서는 90% 이상의 생존율을 나타난 반면, 48시간 후의 경우 73%로 측정되어 시간이 지나면서 방사선에 의한 영향이 세포의 생존 또는 성장을 저해하는 결과로 나타났다. 이와 더불어, 400 Gy 이상에서는 노출 후 배양시간과 무관하게 생존율이 30% 이하로 관찰되었다. 이때 측정된 농도-생존율 값을 이용하여 수식을 도출할 수 있었으며, 수식을 이용하여 처리 후 24시간과 48시간 LD_{50} 값을 구한 결과, 각각 298.03, 255.52 Gy로 산출되었다.

염화수은(Ⅱ) 단독 처리된 세포의 생존율

PLHC-1 세포주를 염화수은 (Ⅱ) 1 ~ 500 uM 농도 범위에서 24시간 및 48시간동안 처리하여 생존율을 관찰하였다. 그 결과, 노출 시간에 상관없이 수은 농도가 증가함에 따라 세포의 생존은 유의하게 감소하였으며, 100 uM 까지는 대조군 대비 90% 이상의 생존율을 나타내었다 (Fig. 37). 이후, 200 uM 이상의 농도에서는 농도 증가에 따라 생존율이 급격하게 감소되어 10% 이하의 생존율이 관찰되었다. 이때 측정된 농도-생존율 값을 이용하여 수식을 도출할 수 있었으며, 수식을 이용하여 24시간과 48시간 노출의 LD_{50}을 구한 결과, 각각 164.12, 158.3 uM로 산출되었다.

방사선과 염화수은(Ⅱ)의 복합처리에 의한 세포의 생존율 변화

방사선과 염화수은 (Ⅱ)에 복합처리된 세포의 생존율 측정에서는 두 가지 요인의 자극 강도가 강해짐에 따라 생존율이 현저하게 감소됨을 알 수 있었다 (Fig. 38). 수은 처리 후 방사선을 처리한 세포의 경우, 방사선 단독 처리군과 비교하여 세포 생존율이 낮게 측정되었으며, 이러한 결과는 각 물질의 저농도에서 더욱 뚜렷하게 관찰되었다. 한편, 방사선과 수은의 복합처리 시 측정된 영향 수치는 각 물질 단독처리 영향의 합보다 크게 나타났다. 이는 방사선과 수은의 복합처리 시 관찰된 세포의 생존율 감소에 대하여 단순 가산이 아닌 상승효과가 나타났음을 의미하는 것으로 유추해 볼 수 있었다.

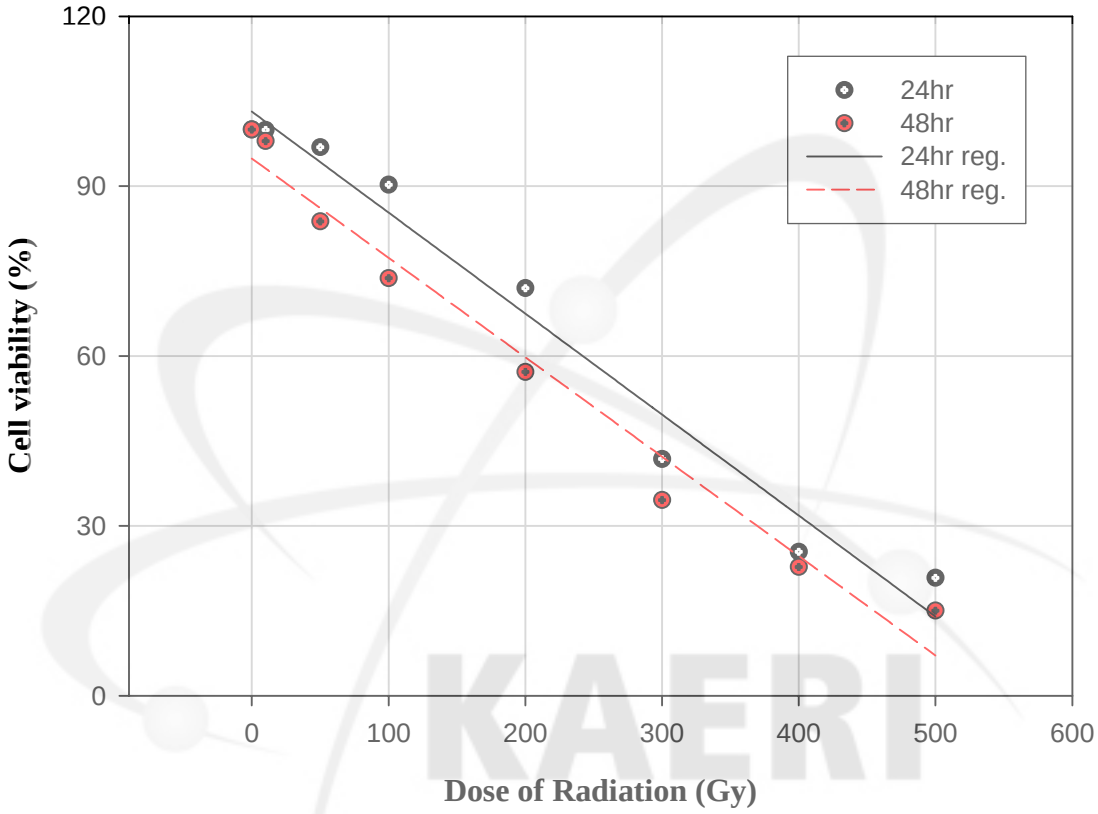

Fig. 36. Effect of the radiation on viability of PLHC-1 cells *in vitro*. Cell viability was assessed by MTT assays after 24, 48 hr (n=3). Data are presented as the percentage of control viability. Cell viability was greatly reduced in a dose- and time-dependent manner by radiation exposure. All the points showed a statistically significant difference from the control group according to Student's *t*-test ($p < 0.005$).

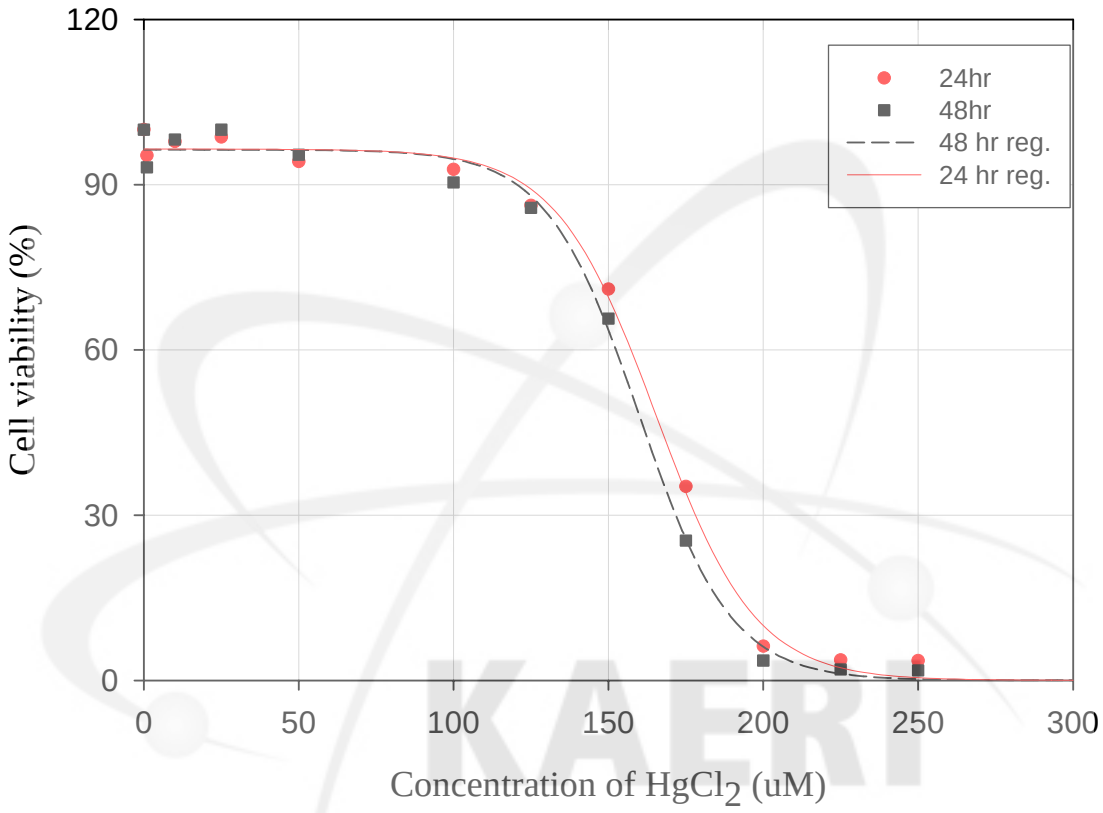

Fig. 37. Effect of the radiation on viability of PLHC-1 cells *in vitro*. Cell viability was assessed by MTT assays after 24, 48 hr (n=3). Data are presented as the percentage of control viability. Cell viability was greatly reduced in a dose- and time-dependent manner by $HgCl_2$ exposure. All the points showed a statistically significant difference from the control group according to Student's *t*-test ($p < 0.005$).

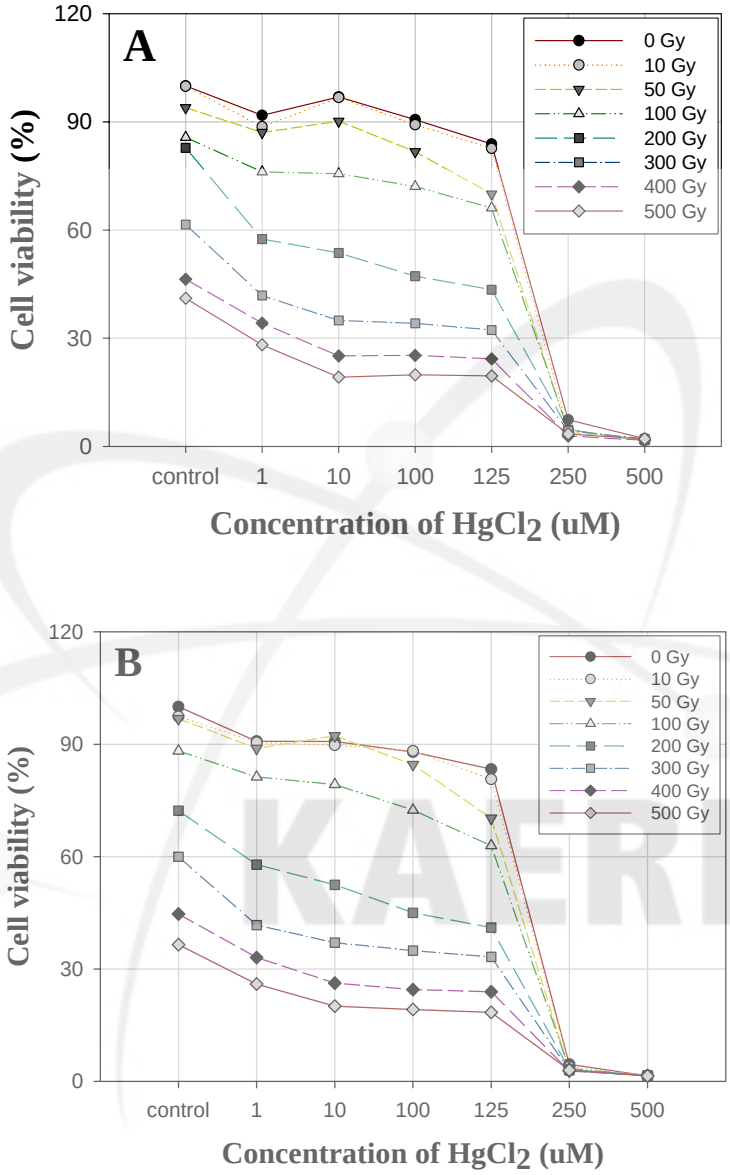

Fig. 38. The *in vitro* cytotoxicity assays of the irradiation and $HgCl_2$ treatment on PLHC-1 cells. Cytotoxicity was measured by MTT assay after 24 hr (a) and 48 hr (b) exposure, respectively (n=3). Cell viability was greatly reduced in a dose-dependent manner by radiation and $HgCl_2$ exposure. All the points showed a statistically significant difference from the control group according to Student's *t*-test ($p < 0.005$).

4. 고 찰

방사선 자극에 대한 생물 반응 연구의 범위가 점차 넓어지고 있지만, 방사선의 단독처리에 의한 영향 연구를 중심으로 수행되어 왔기 때문에 타 물질간의 복합처리에 따른 생물체내의 상승, 가산, 길항작용 등 복합작용 규명과 관련한 연구가 필요한 분야라고 할 수 있다. 또한, 이온화 방사선과 화학물질 등의 단독노출에 대한 연구라 하더라도 주로 사람을 포함한 포유류를 중심으로 이루어져 왔으며, 실제 환경에서 직접 노출되는 생물종에 대해서는 아직 연구가 부족한 실정으로 환경생물종의 연구를 통하여 포유류의 연구 결과와 비교가 가능하다. 환경생물종 중에서도 어류는 다양한 분야에서 널리 이용되며, 그중에서도 어류세포는 신속하고 실험 용이한 장점으로 경제적인 정보를 제공할 수 있다 (Kammann *et al*., 2000; Kolpoth *et al*., 1999). 특히, 본 연구에서 사용된 PLHC-1 세포주는 어류간암세포로써 중금속과 같은 스트레스 요인의 *in vitro* 스크리닝 평가 시, 관련 단백질과 세포독성평가 기법을 이용하여 널리 이용되는 세포주 중 하나이다 (Ryan *et al*., 1994). 그 외에도 유기화합물에 대한 어류 세포를 이용한 생태평가 시, 대상생물로써 많은 연구가 진행되었다 (Bruschweiler *et al*., 1995; Fent, 1996). 또한, PLHC-1 세포주 등 어류 세포를 이용한 독성평가기법은 단시간의 결과 도출과 경제성이 높기 때문에 생태독성평가 시 예비시험 기법으로써의 활용성이 높다고 알려져 있다 (Kammann *et al*., 2000; Kolpoth *et al*., 1999).

따라서, 본 연구에서는 PLHC-1 세포주를 이용하여 방사선 자극에 대한 평가의 적합성 유무 확인과 더불어, 이온화 방사선과 수은에 동시처리 시 나타나는 손상에 대하여 생존율 변화를 통해 관찰하였다. 방사선 및 수은 단독 처리군에서는 농도가 증가함에 따라 세포의 생존은 유의하게 감소하였다. 이때, 측정된 농도-생존율 수식에 의해 처리 24, 48시간의 LD_{50}는 각각 298.03, 255.52 Gy로 산출되었다. 이와 더불어, 방사선 선량 300 Gy 까지는 처리 후 24시간보다 48시간의 생존율이 10 ~ 15% 가량 낮게 나타나 시간이 지나면서 방사선에 의한 영향이 세포의 생존 또는 성장 저해 결과로 나타났음을 확인할 수 있었다. 어류세포의 경우, 방사선 자극에 대한 방어 효과 반응에서는 포유류와 비교하여 다른 경향을 보였지만 (Ryan *et al*., 2008), 실제 자극 반응 실험에서는 처리 선량 및 시간 등과 관련하여 포유류의 연구 범위 내 적용 가능성이 보고된 바 있다 (Kurihara *et al*., 1992). 한편, 염화수은 (Ⅱ)에 단독 처리된 세포의 경우 방사선과 마찬가지로 세포의 생존이 유의하게 감소하였다. 24, 48시간 처리의 LD_{50} 값은 각각 164.12, 158.3 uM로 사람간암세포의 LD_{50} (24 hr) 값이 약 90 ~ 100 uM인 것 (Lee *et al*., 2009)과 비교하여 다소 둔하다고 볼 수 있지만, 본 연구의 결과를 통해 어류세포를 이용한 생태영향평가 및 사람세포와의 비교 가능성을 확인할 수 있었다.

방사선과 염화수은 (Ⅱ)에 복합처리된 세포의 생존율 측정에서는 두 가지 요인의 자극 강도가 강해짐에 따라 생존율이 현저하게 감소되었으며, 각 요인의 상호작용에 의한 영향이 이론적인 단순가산값 보다 높게 나타나 상승작용으로 확인되었다. 본 연구 결과 이외에 방사선과 타 화학물질간의 상승작용은 사람 림프구 및 자궁경부암 세포를 대상으로 수은과 이온화 방사선의 동시처리 시 나타나는 DNA 손상 및 수복기작에 대하여 연구된 바 있다 (Panek *et al*., 2001; Woo *et al*., 2006). 수은 이외 물질 중 살충제와 방사선을 사람의 림프구 세포에 동시 처리시켜 상승작용을 관찰하는 등 복합작용 연구가 시도되었으며(Kim, 2001), 이는 제한적인 결과만을 제시해준 만큼 더욱 넓은 범위의 연구가 필요하다고 볼 수 있다.

본 연구결과들을 통해 방사선 자극에 대하여 PLHC-1 세포주를 이용한 평가의 가능성을 시사해 줌은 물론, 방사선과 염화수은 (Ⅱ)에 복합처리된 세포의 생존 변화로써 각 물질의 상승작용을 확인할 수 있었다. 이와 더불어, 이온화 방사선과 타 물질간의 복합작용 연구 시 세포의 생존뿐만 아니라 세포내 효소활성 변화, DAN 손상 정도 측정 등 다양한 생체지표를 이용하여 연구를 수행한다면 방사선 생태영향평가의 기초이론 정립을 위한 자료로써 활용될 수 있다고 판단된다.

5. 결 론

방사선 자극 평가 시 PLHC-1 세포의 적합성 확인과 함께, 방사선과 염화수은 (Ⅱ)의 단독처리 및 복합처리 시 나타나는 손상에 대하여 관찰하고자 본 연구를 수행하였다. 방사선과 수은의 단독처리군의 경우, 생존율에 따른 LD_{50} (24 hr) 값은 각각 298.03 Gy, 164.12 uM로 산출되었다. 방사선과 수은의 복합처리에 의한 실험 결과에서는 두 가지 요인의 자극 강도가 강해짐에 따라 생존율이 현저하게 감소되었으며, 각 요인의 상호작용에 의한 영향이 이론적인 단순가산값 보다 높게 나타나 상승작용으로 확인되었다. 이러한 결과는 기존 사람세포를 대상으로 한 연구와 유사한 경향을 나타낸 것으로써 생존율을 비롯하여 타 생체지표를 이용한 결과와 함께 병행하여 더욱 정확한 결과를 도출할 수 있을 것으로 사료된다.

제5절 방사선 생물영향 평가 및 검출기법 개발 : Cell Survival and Gene Expressions after Treatment of Mercury Chloride (Ⅱ) and Ionizing Radiation

1. INTRODUCTION

Yeast is a commonly used material in biological research. *S. cerevisiae* is an ideal model organism for deducing biological processes in human cells. Completions of the human and yeast genome sequences have considerably increased the opportunities to address a human gene function using yeast. All aerobically growing organisms suffer from exposure to oxidative stress, caused by partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS). These are highly reactive and capable of damaging cellular constituents such as DNA, lipids and proteins. Consequently, cells from many different organisms have evolved mechanisms to protect their components against ROS. ROS can also be formed by exposure of cells either to ionizing radiation or redox-cycling chemicals present in the environment like heavy metals (Jamieson, 1998). Many diseases and most of the genes associated with the repair of radiation-induced damage in mammalian cells were initially characterized in yeast. Ionizing radiation is used for therapy, diagnosis, prevention of diseases, and sterilizing of foods. Exposure of cells to DNA-damaging agents like ionizing radiation (IR) results in complex response mechanisms to maintain a genetic integrity after DNA damage. These include a cell cycle delay, repair of DNA damage, transcriptional responses, and a programmed cell death. Because a disruption of any one of these DNA damage responses can lead to a genomic instability and cancer, a comprehensive understanding of the individual components and their regulation is crucial (Bennett *et al*., 2001; Watson *et al*., 2004). The most biologically relevant lesions are DNA double strand breaks (DSBs). Approximately 30 genes have been implicated in the repair of radiation-induced DSBs by recombination and/or nonhomologous end-joining repair (NHEJ) pathways in yeast. Mating type regulation can influence the affect of these genes on a cell-cycle arrest in opposite ways. They are part of the adaptation process in haploids (possibly by attenuating the checkpoint response), whereas in diploids they cause a cell-cycle arrest (part of the checkpoint

response mechanism). DSBs in haploid yeast are repaired primarily by a homologous recombination between sister chromatids in the S or G_2 phase of a cell cycle; in diploid cells, recombinational repair can also occur between homologous chromosomes throughout a cell cycle. The NHEJ pathway, a relatively minor pathway for a repair of radiation-induced DSBs in yeast, is responsible for the repair of most DSBs induced in mammalian cells.

DNA damage checkpoint and pathway are associated with two highly conserved phosphoinositol-related kinases, *Ataxia telangiectasia* mutated (ATM), ATM and Rad3-related (ATR) and their yeast homologs, *Sp*Tel1 and *Sp*Rad3 in *S. pombe*, and *Sc*Tel1 and *Sc*Mec1 in *S. cerevisiae*. Activation of ATM and ATR kinases by DNA damage leads to a cell cycle arrest through a number of downstream effector molecules including the effector kinases CHK1/*Sp*Chk1/*Sc*Chk1 and CHK2/*Sp*Cds1/*Sc*Rad53. In addition to regulating a cell cycle, DNA damage checkpoints also coordinate transcriptional responses that facilitate in a DNA synthesis and repair (Watson *et al*., 2004).

Metal ions are essential to life. However, some metals such as mercury are harmful, even when present at trace amounts. Toxicity of mercury arises mainly from its oxidizing properties. As a metal ion, it induces an oxidative stress or predisposes cells to an oxidative stress, with considerable damage to proteins, lipids and DNA.

The protective effect of Ycf1p against the toxicity of mercury is especially pronounced when yeast cells are grown in a rich medium or in a minimal medium supplemented with glutathione. Secretory vesicles from *S. cerevisiae* cells induce the expression of Ycf1p and a transport by this protein of mercury-glutathione adducts into the vacuole (Gueldry *et al*., 2003). Ycf1 is positively regulated by distinct, but related bZIP transcriptional activators, Yap1 (Bouganim *et al*., 2001). *YCF1* gene encodes an ABC transporter protein that shares a strong sequence similarity with a human cystic fibrosis transmembrane conductance regulator and a multidrug resistance-associated protein. *YCF1* is a target gene for a yAP-1 transcriptional control (Wemmie *et al*., 1994).

Yap (yeast activator protein) induced activities of several enzymes involved in an oxygen detoxification. Yap1 is required for the stress induced expression of *TRX2*, encoding thioredoxin and a *TRR1* encoding thioredoxing reductase (Mark Toone and

Jones, 1998; Carmel-Harel *et al.*, 2001). Yap1 also controls the expression of certain ABC transporter protein encoding genes, namely *YCF1*, involved in the removal or sequestration of cadmium (Mark Toone and Jones, 1998). In the absence of a stress, Yap1p is present in both the cytoplasm and the nucleus. Upon a treatment with an oxidative stress, the protein is concentrated in the nucleus.

The mechanism governing this stress-dependent relocalization operates at the level of a nuclear export and involves the cysteine-rich-domain (CRD) in the C-terminus of the Yap1p which results in a constitutive nuclear protein with concomitantly high expression levels of a Yap1 reporter construction. The C-terminal cysteine-rich domain (c-CRD) of Yap1p, which contains the nuclear export signal (NES), is responsible for this regulated interaction with Crm1p (Mark Toone and Jones, 1998; Carmel-Harel *et al.*, 2001; Kuge *et al.*, 2001; Wysocki *et al.*, 2004). Yap1p localization is controlled by Crm1p, ybp1p, and a Gpx mediated nuclear export and that was nuclear export signals within both the n-CRD and the c-CRD. Carmel-Harel *et al.* (2001) revealed that the glutathione peroxide-like protein Gpx3p is required for a Yap1p oxidation by H_2O_2 (Carmel-Harel *et al.*, 2001; Wysocki *et al.*, 2004). Metalloid exposure triggers a nuclear accumulation of Yap1p and stimulates an expression of antioxidant genes. Yap1p is being regulated by a metalloid stress which indicates that this activation of Yap1p operates in a manner distinct from a stress caused by chemical oxidants (Wemmie *et al.*, 1997; Wysocki *et al.*, 2004). The first line of defense during the metalloid exposure is an increase in a GSH synthesis coupled to a Ycf1p activity as well as an oxidative stress response. When the exposure persists, expression of the detoxification system *ACR3* is stimulated to ensure a continuous metalloid removal from the cytosol (Wysocki *et al.*, 2004). Yap1p mediates the cadmium tolerance by controlling the *YCF1* expression (Carmel-Harel *et al.*, 2001; Wysocki *et al.*, 2004).

In yeast, the physiological response to changing environmental conditions is controlled by a cell type. The previous study on the cell viability after treatment of radiation and mercury resulted in different response to the same stress condition in both cell types (haploid and diploid) (Kim *et al.*, 2007). Diploids are more resistant than haploids to a radiation stress. They are also more resistant to a heavy metal (mercury, cadmium, etc.) (Mercier *et al.*, 2005; Valencia-Buton *et al.*, 2006). Haploid cells were used in this experiment, namely *S. cerevisiae* strain W303-1A *MAT*a

{*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*}. W303 strain contains a mutant allele of *YBP1*, *ybp1-1*, encoding four amino acid substitutions. The *YBP1* gene was also resistance to an oxidative stress (Rodrigues-Pousada *et al*., 2004). The expression of the *YAP* and *YCF* genes was investigated by a Real-time PCR and the cell survival induced by radiation and mercury stresses in *S. cerevisiae* was discussed.

2. MATERIALS AND METHODS

Strain and Culture

A *S. cerevisiae* strain W303-1A *MAT*a {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*} was grown on a rotary shaker in a YPD medium with 1% Yeast Extract (Difco), 2% Bacto Peptone (Difco) with 2% glucose at 30℃ for 60 h. For the culture used in the analysis 20 ㎖ of a 24 h grown pre-culture, 6 ㎖ of a pre-culture medium was transferred to a 1 liter flask containing a 300 ㎖ fresh medium. Growth was checked spectrophotometrically by measuring the optical density at 600 nm.

CFU (colony forming unit)

Cell viability was measured by means of CFU (colony forming unit). The culture approached an optical density (600 nm) equal to 0.6, which corresponds to a density of 2.9×10^7 cells $m\ell^{-1}$. Plating cells in the corresponding medium were supplemented with 15 g of agar per liter. The cells were diluted and spread on the plate treated with $HgCl_2$ or without $HgCl_2$. Experiments were performed three times and the cell survival rates were calculated from an average of the three experimental data.

Stress induction

Metalloid stress was induced by liquefied $HgCl_2$. Treatment of mercury chloride (II) was done in the concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mM. For an oxidative stress induced by ionizing radiation, 100, 400, 800 and 1200 Gy hr^{-1} were applied to obtain a total dose of 400 and 800 Gy, respectively. A combined

stress was induced with a medium containing mercury chloride (II) after the cells were irradiated with ionizing radiation.

RNA isolation

S. cerevisiae was grown on a complex medium of YPD to the logarithmic growth phase. Total RNA was extracted using the Ribo-Pure Yeast (Ambion) following the protocol provided with the kit. DEPC-treated water was used in the process. The extracted RNA was quantified by an absorbance measurement at 260 nm. Some mediums were treated with mercury.

Real-time PCR

The cDNA for the Real-time analysis was prepared with ycf_RT_R, yap_RT_R and act_RT_R primers by RT-PCR reactions. The Real-time PCR analysis was performed using the SYBR Premix Ex Taq (TaKaRa), with the prepared cDNA (2 μg reaction^{-1}) and primers; ycf_F2/R2, yap_F2/R1, act_F3/R2 (Table 10). Cycling conditions were as follows : (1) Initial denaturation for 10 sec at 95℃ ; (2) Denaturation for 5 sec at 95℃ ; (3) Annealing for 20 sec at 52℃ ; (4) Extension for 20 sec at 72℃ ; (5) dissociation for 15 sec at 95℃, for 30 sec at 60℃, for 15 sec at 95℃. The 2nd ~ 4th steps were repeated for 40 cycles. Data collection was performed at the 4th , and 5th steps.

3. RESULTS AND DISCUSSION

Metalloid and ionizing radiation could induce a kind of oxidative stress. Total doses of ionizing radiation treatments were 100, 400, and 800 Gy for each dose rates. Cell survival rates were 74.8%, 65.6%, 55.0% after irradiation with the lowest dose rate of 100 Gy hr^{-1}, and 59.1%, 50.4%, 37.4% with the middle dose rate of 800 Gy hr^{-1}. The survival rates of the cells irradiated with the same dose of radiation were different depending on dose rates. The cell viability reduced more with the higher

dose rates (800 and 1200 Gy hr^{-1}) than with the lower dose rates (100 and 400 Gy hr^{-1}). The viability of the irradiated cells was prone to be inversely proportional to a radiation dose rate and a total dose (Table 11).

Treatment of mercury chloride (Ⅱ) was done in the concentration range from 0.1 to 0.9 mM. The cell survival rates significantly reduced in a concentration higher than 0.2 mM. The half-lethal concentration estimated from the survival curve was 0.253 mM. The cells were almost extinct when the concentration of the mercury chloride (II) exceeded 0.3 mM. The cells treated with low concentration of mercury chloride (II) showed higher survival rate (Fig 39). The cells treated only with mercury chloride (Ⅱ) were inactivated in concentrations over 0.4 mM (Fig 39).

The combined treatment of 100 Gy with 0.2 mM or 0.25 mM of mercury chloride (II) significantly reduced the survival rates of the cells, while the combination of 100 Gy with 0.3 mM resulted in extinction of the cells (Fig 40). The combination of ionizing radiation 800 Gy with 0.3 mM $HgCl_2$ caused a complete inactivation of the cells. Simultaneous treatment of the two stressors resulted in a lower viability than that of one stressor only. In other word, the combined treatment affected, in terms of viability, the cells more than a single stress treatment.

The *YCF* gene mutated strain became extinct when treated with 0.25 mM cadmium. But, the *YAP* mutated strain grew better in the cadmium medium than the *YCF* mutated cells (Ono *et al.*, 1988). The expression of the *YCF* gene increased at a higher total dose (Fig 41. B). The expression of a particular gene was dependent on kinases, the mean expression levels exhibited a $\geq$ 2-fold change in an expression in the mutant compared to a wild-type after exposure to ionizing radiation (Watson *et al.*, 2004). For the mechanism of protection against a radiation-induced lethality, several gene groups are implicated indirectly through the mechanisms of mRNA and protein stability and/or trafficking. Identification of genes controlling DNA-damage responses in yeast has often led to the discovery of functionally related genes in mammalian cells, including many DNA repair genes (Bennett *et al.*, 2001). The 0.0 mM, 0.25 mM and 0.3 mM treated cells revealed the same expression of the *YCF* gene (Fig 41. B). The expression of *YCF1* is induced by the presence of cadmium or by limiting levels of adenine. The response of *YCF1* to cadmium is mediated by an up-regulation of Yap1p, a transcriptional factor which positively controls the expression of numerous genes involved in an adaptation to an oxidative stress. A

Yap1p-regulatory element was identified in the *YCF1* promoter region. The *YCF1* strain corresponds to stoichiometries of mercury to glutathione close to 1:2. Up to this stoichiometry value, additional mercury is expected to become fully trapped in an $Hg(GS)_2$ complex. Therefore, the protection conferred by glutathione in the *YCF1* context remains efficient as long as all the mercury is converted into $Hg(GS)_2$. Moreover, the strong protection associated with the presence of functional Ycf1p strongly suggests that $Hg(GS)_2$, once in a cell, is transported into the vacuole. Cadmium, mercury or arsenate additions have similar consequences on the activity of the *YCF1* gene (Gueldry *et al.*, 2003). *S. cerevisiae* is capable of launching an adaptive stress response to mercury. There are some overlaps between the mercury and H_2O_2-adaptive stress responses. Mercury regulates a *GSH1*gene expression by a mechanism distinct from that used by cadmium (Westwater *et al.*, 2002). Cadmium is compartmentalized in vacuoles through the Ycf1 transporter, in the form of a $Cd(GS)_2$ complex. The influx system (represented by the Zrt1 protein), glutathione and Ycf1 (a vacuolar glutathione S-conjugate pump) control the cadmium homeosis (Gomes *et al.*, 2002).

The results from the cells treated only with mercury chloride (Ⅱ) were compared to those from the cells treated with ionizing radiation and mercury chloride (Ⅱ) at the same time (Fig 40). The *YCF* gene expression was lower in the cells irradiated with 400 Gy than that of the non-irradiated control cells. In particular, the cells irradiated with 400 Gy showed a lower expression of *YCF* gene than the cells irradiated with 800 Gy (Fig 41. B). The *YCF* gene expression, however, showed a different pattern in the 0.2 mM $HgCl_2$ treated cells. The *YCF* gene expression was higher in the cells with the simultaneous treatment of radiation and mercury chloride (II) than the cells treated with mercury chloride (II) only, while the *YAP* gene expression was higher in the cells treated with mercury chloride (Ⅱ) only. The cells irradiated with 400 Gy resulted in a higher expression of the *YCF* gene than the cells irradiated with 800 Gy at both dose rates. The 800 Gy irradiated cells revealed a lower *YCF* gene expression than the 400 Gy irradiated cells at the 400 Gy hr^{-1} dose rate. In case of 800 Gy hr^{-1} dose rate, the *YCF* gene was expressed in the cells irradiated with 400 Gy and 800 Gy, as well (Fig 41. B). A lower expression level was found in the 0.2 mM $HgCl_2$ treated cells than in the non-treated cells. However, the expression level increased with the concentration of mercury chloride

(Ⅱ). Various oxidants activate Yap1p and this protein is required for an oxidative stress tolerance as well as for a resistance to heavy metals or cytotoxic agents. Yap1 positively regulates the expression of several genes including *YCF1*, *GSH1* encoding ɣ-glutamylcysteine synthetase (involved in the biosynthesis of antioxidant glutathione), *TRX2* encoding antioxidant thioredoxin, and *GLR1* encoding glutathione reductase (Bouganim *et al.*, 2001; Gueldry *et al.*, 2003). Transcription factor Yap1p plays a major role in conferring a cellular resistance to mercury and also in regulating the *GSH1* expression in response to this metal. This finding is consistent with the epistatic relationship described above and is presumably due to a direct or indirect enhancement of the *YCF1* gene transcription by yAP-1. The expression of *YCF1* positively correlates with changes in the *YAP1* gene dosage. The observed yAP-1 dependent changes in *YCF1* mRNA are brought about through an action of yAP-1 on the *YCF1* promoter region (Wemmie *et al.*, 1994). Mercury chloride (II) treated cells except for 0.2 mM showed a higher *YAP* gene expression for 800 Gy than 400 Gy total dose at both dose rates. Also the cells irradiated at lower dose rate showed a lower expression of the *YAP* gene. In other words, the expression of the *YAP* gene increased under the condition of low dose rate and high total dose. However, for the 0.2 mM $HgCl_2$ treated cells, the expression of the *YAP* gene increased at the high dose rates and with a low total dose (Fig 41. A).

Exceptionally, both genes in the 0.2 mM $HgCl_2$ treated cells were expressed more when irradiated with 400 Gy than the 800 Gy total dose where the dose rate was 400 Gy hr^{-1}. At 800 Gy hr^{-1} dose rate, a greater gene expression was induced than at the 400 Gy hr^{-1} dose rate. The results meant that a lower total dose increased the expression of the genes.

4. CONCLUSIONS

The survival rate of cells irradiated with a fixed dose was different depending on the dose rate. The cell survival decreased with dose rate, and with mercury concentration, as well. The higher the dose rate was, the lower the cell survival rate was. And the viability of the cells treated with mercury chloride (Ⅱ) higher than 3 mM came down to zero. The cell viability was reduced significantly by the treatment of mercury chloride (Ⅱ) combined with ionizing radiation. The combined treatment

had a greater impact on the cell survival. *YCF* mRNA level positively correlated with changes in the *YAP* gene dosage (Wemmie *et al.*, 1994). In the present work, the *YCF* gene expression was more induced by a stressor than the *YAP* gene. The expression of both the *YAP* and *YCF* genes was particularly lower in the cells treated with 0.2 mM $HgCl_2$ compared to that of the cells treated with any other concentration else.

Both the *YAP* and *YCF* genes expressions were considerably induced in the concentration of higher than 0.25 mM $HgCl_2$. The expression of the genes increased under the higher total dose. The *YAP* and *YCF* genes expression induced by a metalloid stress increased with mercury chloride (Ⅱ) concentration. Ionizing radiation caused both the *YAP* and *YCF* genes expression caused by ionizing radiation increased with the total dose. But, both the cells treated with 0.2 mM $HgCl_2$ and the cells irradiated with ionizing radiation displayed a higher cell survival rate in spite of a low expression of the stress resistance genes. It would be important to examine the role of mercury and ionizing radiation stress on cell survival responses. In case of combined treatments, the *YAP* and *YCF* genes expression induced by a metalloid stress increased with $HgCl_2$ concentration and with the total dose of ionizing radiation, as well. The combined treatment resulted in synergistic increase in gene expressions, and the increase was found to be dose-dependent.

Table 10. Primer sequences used in PCR and Real-time PCR

Primers	Sequence of primers
ycf_F2	5′- AGTAATAAGGTGAGCGCGTTATCCATCGCA -3′
ycf_R2	5′- GCCCTCCTTAAACTTATGGCGTCAGAGTTG -3′
yap_F2	5′- CGAAAATGAACCAGGTATGTGGAACAAGGC -3′
yap_R1	5′- CGAAACCAAGTCGGCCAAAACCGGAGAAGG -3′
act_F3	5′- TTGAACACGGTATTGTCACCA -3′
act_R2	5′- AAACAATACCAGTTCTACCGG -3′
ycf_RT_R	5′- TTAATTTTCATTGACCAAACCAGC -3′
yap_RT_R	5′- CTAATTGAACGTCTTCTGCA -3′
act_RT_R	5′- ACTTGTGGTGAACGATAGATGGACCACTTT -3′

Table 11. Cell viability (%) of *S. cerevisiae* cells irradiated with ionizing radiation

		Dose rate (Gy hr^{-1})			
		100	400	800	1200
Total Dose (Gy)	0	100.0	100.0	100.0	100.0
	100	74.8	49.5	59.1	47.7
	400	65.6	73.6	50.4	48.9
	800	55.0	50.4	37.4	38.8

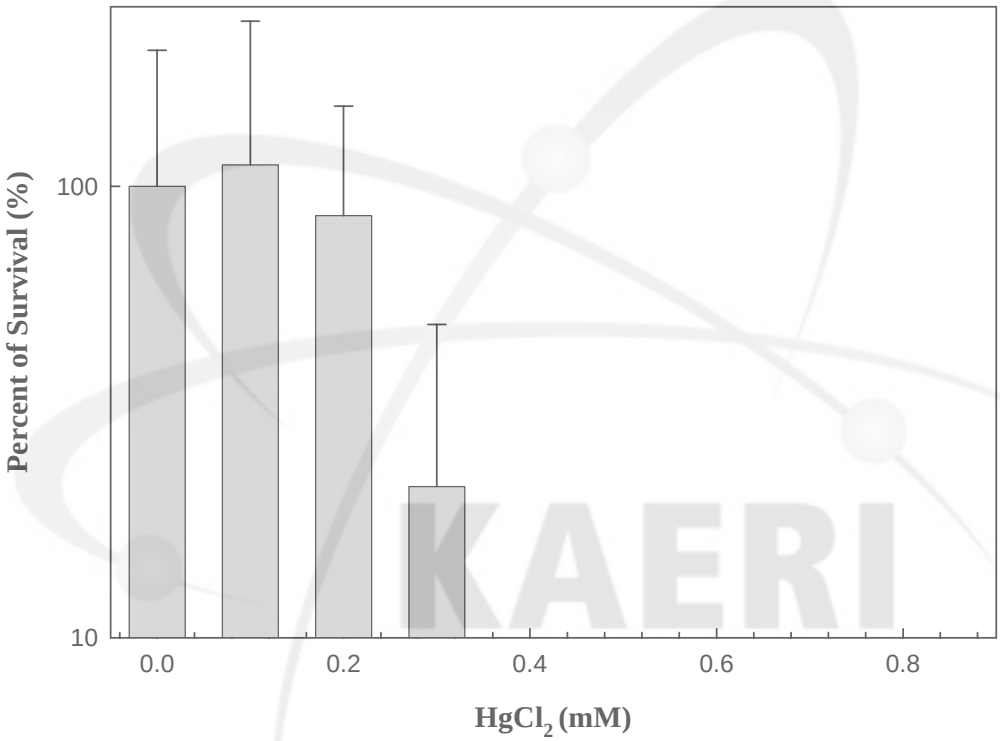

Fig. 39. Cell viability of *S. cerevisiae* treated with various concentrations of mercury chloride (II).

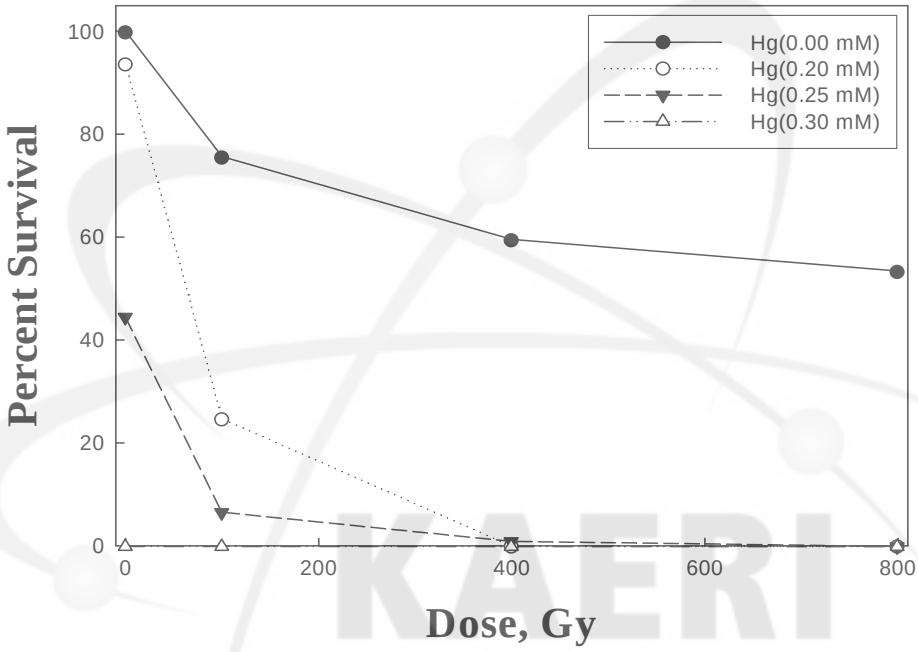

Fig. 40. Cell survival rate of *S. cerevisiae* after treatment of mercury chloride (Ⅱ) combined with ionizing radiation (dose rate; 400 Gy hr^{-1}).

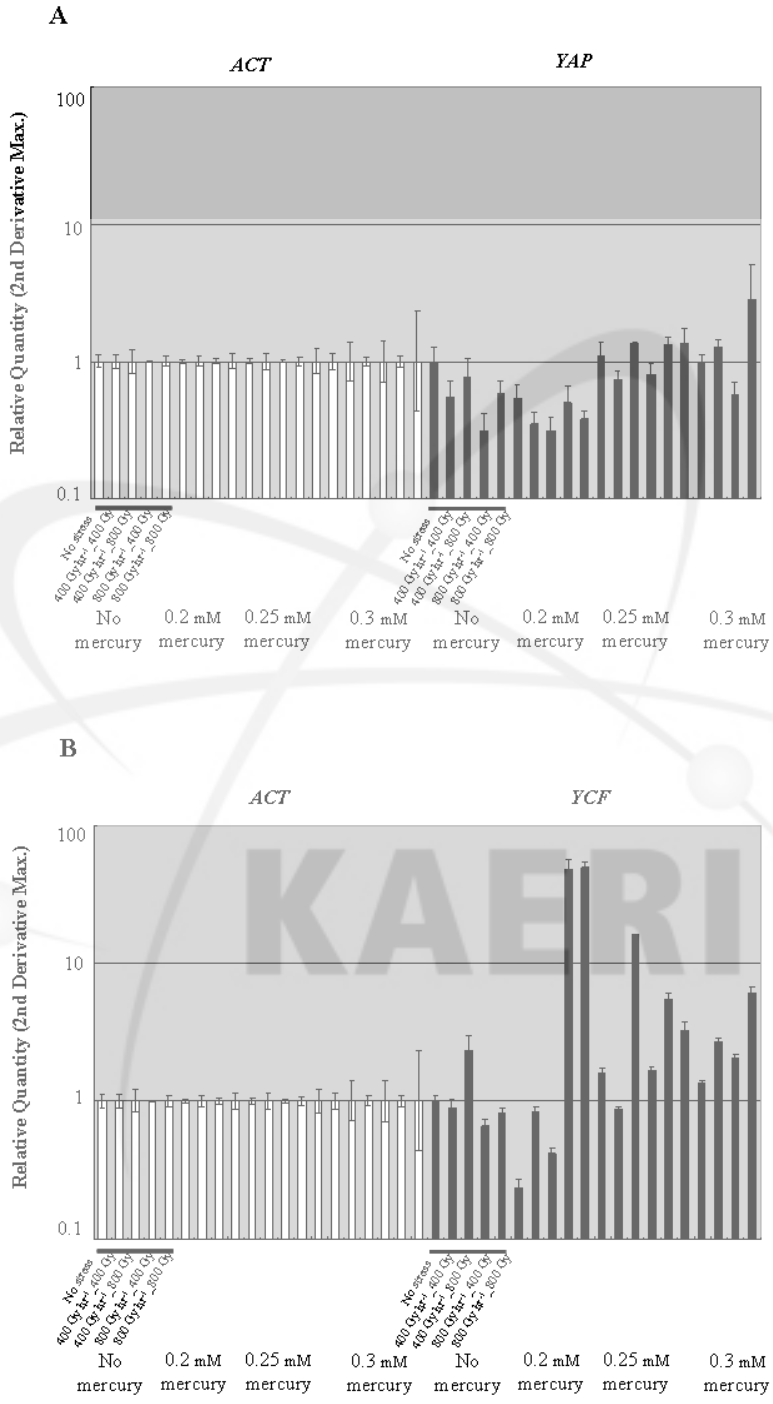

Fig. 41. Gene expressions of *YAP* (A) and *YCF* (B) assessed by the Real-time PCR

제6절 방사선과 복합처리조건에 따른 상승작용 이론 수립 및 검증

1. INTRODUCTION

Ecosystems and living organisms have never been exposed to merely one harmful agent. Many physical, chemical, biological and social factors may simultaneously exert their influence to organisms and the environment. In many examples of interactions between ionizing radiation and other toxic physical or chemical agents, the biological responses range from strong antagonism to strong synergism. The most effective simultaneous exposure to ionizing radiation and another factor may result in four possible modes of interaction: (i) no interaction, the effects observed are based on the most toxic agent; (ii) additivity, the effects produced by separate application of each agent are simply summed up; this mode can be subdivided on the additive and independent interaction; (iii) antagonism, the effect is less than expected additivity; (iv) synergism or supra-additivity, the effect is greater than expected additivity.

The combined exposure to two harmful agents could result in a higher effect than would be expected from the addition of the separate exposures to individual agents (UNSCEAR 1982; Streffer and Müller, 1984; UNSCEAR 2000). Hence, there is a possibility that, at least at high exposures, the combined effect of ionizing radiation with other environmental factors can result in a greater overall risk. The problem is not so clear for low intensity and there is no possibility of testing all conceivable combinations of agents. Moreover, there are contradictions in literatures devoted to synergy problems relative to interaction effectiveness in the dependence of dose of applied agents and their intensity which results in various opinions about the importance of the synergistic interaction at low intensity of harmful agents found in biosphere (Streffer and Müller, 1984; UNSCEAR 2000; Dethlefsen and Dewey, 1982; Kuzin, 1990).

A great number of experimental data obtained in this area stresses the need for a mathematical approach in efforts to optimize and predict the interaction of environmental agents. Recently, a simple mathematical model was proposed to describe the synergistic effect of simultaneous action of ionizing radiation and high temperature (Petin and Komarov, 1997). The basic postulates underlying the model were not related to a particular cytotoxic agent, so the model may have an

application to describe the synergistic interaction of other agents. Some examples have been already published. Our purpose is to discuss the simplest model to account for currently available experimental findings. This work describes a part of continuing research activity directed at optimizing the combined action of two harmful agents by achieving some new insight into the synergistic mechanism. To gain deeper insight into the mode of interaction, we have discussed the following tasks in this paper. (1) To reveal common peculiarities of synergistic interaction display. (2) To study post-irradiation cell recovery after combined actions. (3) To suggest a concept of synergistic interaction for the interpretation of reveled regularities and to formulate a common mathematical model to describe, optimize and predict the synergistic effects. (4) To prove the condition under which the highest or any equieffective values of the synergistic enhancement ratio can be achieved. (5) To compare the model predictions with experimental results. (6) To demonstrate that, for any fixed intensity of one agent, the synergistic effect might be increased, decreased or remain without change with alteration of the intensity of another agent. The results obtained are discussed from the viewpoint of potential significance of synergistic interaction of deleterious agents delivered at intensities occurring in the human environment.

2. EXPERIMERIMENTAL ANALYSIS OF SYNERGISTIC INTERACTION

A lot of experimental data have been obtained for the action of various agents combined with hyperthermia (Petin and Zhurakovskaya, 1995, 1997; Petin *et al.*, 1998; Kim *et al.*, 2001; Petin and Kim, 2002; Kim *et al.*, 2002). For these cases, it was shown that synergistic enhancement ratio increases, reaches the highest value and then drops with increase in the ambient temperature. This dependence suggests that the equieffective synergy may be realized at various temperatures.

Fig. 42 provides an example of the basic experimental data used in this investigation. Four types of survival curves were obtained for every condition of thermoradiation action: a heat treatment alone (curve 1), ionizing radiation (or another physical agent or chemical compound) without heating (curve 2), composite simultaneous heat and radiation exposure (curve 4). Curve 3 represents a theoretically expected survival curve that would be obtained if inactivation by composite heat and

radiation were completely independent. To estimate quantitatively the sensitization action of hyperthermia, one can apply the thermal enhancement ratio (Stewart and Denecamp, 1978), defined as D_3/D_1 (Fig. 42). This ratio indicates an increase in cell radiosensitivity by high temperature. However, it does not reflect the kind of interaction. To evaluate the synergistic effect, the synergistic enhancement ratio, defined as D_2/D_1 (Fig. 42) has been used in various studies (Petin and Zhurakovskaya, 1995, 1997; Petin *et al.*, 1998; Kim *et al.*, 2001; Petin and Kim, 2002; Kim *et al.*, 2002). It is curious that the thermal enhancement ratio increases indefinitely with increasing exposure temperature, while the synergistic enhancement ratio at first increases, then reaches a maximum, which is followed by a decrease. This implies that a synergistic interaction between hyperthermia and ionizing radiation is observed only within a certain temperature range. Noteworthy is the fact that such a dependence of the synergistic effect on temperature under which the exposure occurred was obtained for diploid yeast cells upon the simultaneous combination of hyperthermia with ionizing radiation, UV light, ultrasound, and some chemical inactivating agents. Hence, it can be understood that for a given intensity of physical factors or concentration of chemical agents there should be a specific temperature that maximizes the synergistic interaction. Any deviation of temperature from the optimal value results in a decrease of synergism. Another example of similar synergy pattern is reported by Kim *et al.* (2002).

Here again, the dependence of the synergistic enhancement ratio upon the exposure temperature is depicted for relatively thermosensitive (*Endomyces magnusii*), mildly thermoresistant (*Zygosaccharomyces bailli*) and thermoresistant (*Saccharomyces ellipsoideus*) diploid yeast strains. It is noteworthy that the temperature range strengthening the effect of ionizing radiation has been shifted toward the lower temperatures for temperature-sensitive cell lines.

Thus, it is clear that the synergistic interaction between hyperthermia and other inactivated agents is realized only within a certain temperature range independently of the object analyzed. For temperatures below this temperature range, the synergistic effect was not observed and cell killing was mainly determined by the damages induced by ionizing radiation. For temperatures above this temperature range, the synergistic effect was also not observed but cell killing was chiefly caused by hyperthermia. It follows that for a given intensity of physical factors or

concentration of chemical agents there would be a specific temperature that maximizes the synergistic interaction. Any deviation of the exposure temperature from optimal value will result in a decrease of the synergistic interaction. These results, besides being an important key for searching the synergy, can be considered as an indication of the possibility to optimize and achieve a desirable level of synergy.

On the basis of the results presented the following conclusion should also be valid: for any constant ambient temperature (or concentration of chemical agent) there should be an optimal intensity of ionizing or UV light radiation resulting in the greatest synergy. Some selected experimental results confirming this prediction are presented in Fig. 43.

The data on the simultaneous thermoradiation inactivation of *Bacillus subtilis* spores (Reynolds and Garst, 1970; Reynolds and Brannen, 1973) were used to estimate the dependence of the synergistic enhancement ratio on the dose rate at the exposure temperature of 95℃. The results are depicted in Fig. 43A. It can be seen that for a constant temperature, at which the irradiation occurs, synergy can be obtained within a certain dose rate range. Inside this range an optimal dose rate of ionizing radiation may be indicated, which maximizes the synergy. Very similar results were obtained for inactivation of diploid yeast cells exposed to electron beam from a 25 MeV pulsed linear accelerator at 51℃ (Fig. 43B).

Fig. 43C demonstrates the relationship between the synergistic enhancement ratio and the dose rate of ionizing radiation for a combined effect of lead nitrate and chronic irradiation of *Arabidopsis thaliana* seeds. This relationship was calculated using the experimental results obtained by others (Dineva *et al.*, 1993). The seeds were selected in wild populations grown for five years in places with different levels of radioactive pollution inside the 30 ㎞ zone around the Chernobyl nuclear power station and then treated with lead nitrate (3.39 g/ℓ).

The frequency of mutant embryos and the proportion of lethal embryos were estimated. For this case, the synergistic enhancement ratio was defined as the ratio between the increment of the mutant or lethal embryos after the combined action and the sum of these increments after separate action of each agent. As it can be seen from Fig. 43C, the synergistic effect has a pronounced maximum at a certain dose rate of ionizing radiation.

Fig. 43D shows the relationship between the synergistic enhancement ratio and power flux density after simultaneous action of microwave exposure and high environment temperatures (30℃, curve 1, and 38℃, curve 2) on rabbit heating (Kolganova and Zhavoronkov, 2001). Here again, the synergistic effect has a pronounced maximum at a certain intensity of microwave radiation. The only experimental point obtained for rabbits exposed at 100 ㎽/㎠ and 38℃, shows that the synergistic enhancement ratio was increased for this power flux density. It turned out that further investigation of the synergy for lower intensities of microwaves delivered at 38℃ was fruitless because animals did not sustain this high environmental temperature for a long time.

Nevertheless, this data show that the relationship between the synergy and the power flux density may be shifted to higher intensities with an increase in ambient temperature. Then it can be expected that as the exposure temperature is reduced, the optimal dose rate decreases and *vice versa*. The universality of this important conclusion is supported by the author's extensive data with diploid yeast cells exposed by heat combined with ionizing radiation, 254 nm UV light and ultrasound.

The synergistic interaction may increase, decrease or stay unchanged with the decrease in the intensity of any physical factor combined with heat. Nevertheless, the equieffective values of the synergistic interaction and the whole temperature range, within which the synergy can occur, are shifted to a lower temperature. To demonstrate a potential significance of synergistic interaction at low intensity of agents applied, the correlation between the intensity of physical factor or the concentration of chemical compound and the exposure temperature, which both provide equieffective levels of synergistic interaction (Kim *et al.*, 2001). To estimate this correlation, original data were taken from a number of publications for simultaneous thermoradiation action on bacterial spores (Reynolds and Garst, 1970; Reynolds and Brannen, 1973), phage (Trujillo and Dugan, 1972), mammalian cells (Ben-Hur *et al.*, 1974; Ben-Hur, 1976), and rabbit heating (Kolganova and Zhavoronkov, 2001). Original data for mammalian cells exposed to heat combined with cis-DDP or TAPS were taken from the papers (Urano *et al.*, 1990) and (Johnson and Pavelec, 1973), respectively. Linear relationships are found between these values for various biological objects. In all cases, at a smaller intensity of the physical factor or the concentration of the chemical agents, one has to reduce the

acting temperature to preserve the highest or any arbitrary synergistic effect. These data, in principle, indicate a potential significance of synergistic interaction at low intensity of adverse factors encountered in the natural environment.

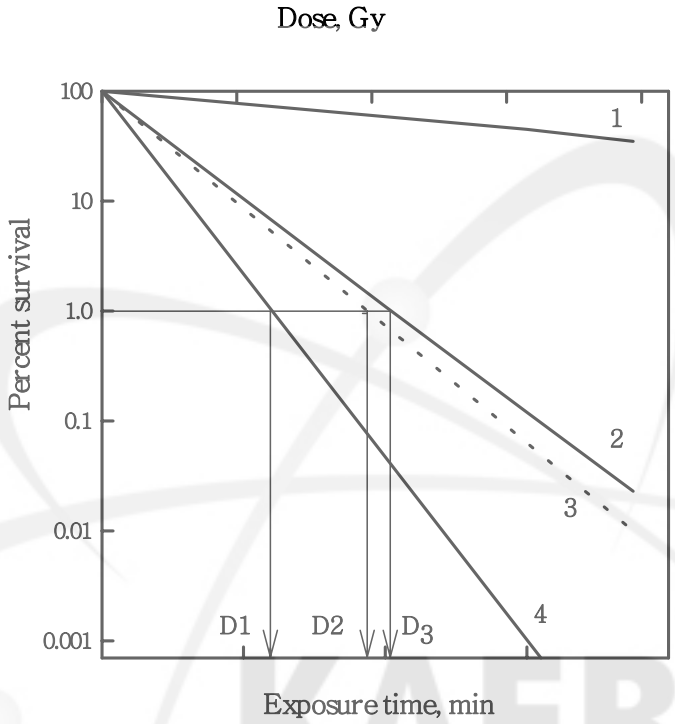

Fig. 42. Example of the basic experimental data. A. Survival curves of haploid yeast cell: curve 1 – heat treatment (45℃) alone; curve 2 – ionizing radiation (^{60}Co) at about 10 Gy/min and room temperature; curve 3 – calculated curve for independent action of ionizing radiation and heat; curve 4 – experimental curve after simultaneous thermoradiation action.

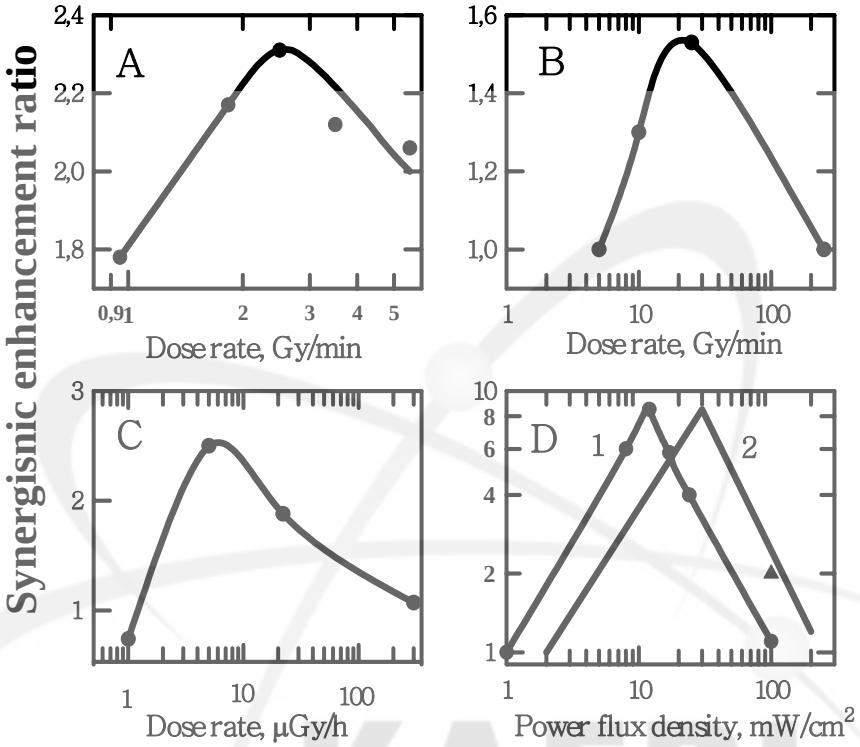

Fig. 43. The dependencies of the synergistic enhancement ratio upon the intensity of physical factors. The role of dose rate for inactivation of bacterial spores *Bacillus subtilis* (A), diploid *Saccharomyces cerevisiae* yeast cells (B) exposed to ionizing radiation at 95 and 51℃, respectively. C – the relationship between the synergistic enhancement ratio and the dose rate of ionizing radiation for a combined effect of lead nitrate and chronic irradiation of *Arabidopsis thaliana* seeds. D – the relationship between the synergistic enhancement ratio and power flux density after simultaneous action of microwave exposure and high environment temperatures (30℃, curve 1, and 38℃, curve 2) on rabbit heating.

3. CELL RECOVERY AFTER COMBINED ACTIONS

The inhibition of cell recovery is commonly considered as a reason of synergy of the combined action of ionizing radiation with various agents (UNSCEAR 1982; Streffer and Müller, 1984; UNSCEAR 2000; Dethlefsen and Dewey, 1982). However, the inhibition may be proceeded *via* either the damage of the mechanism of the recovery itself or *via* the formation of irreversible damage which can not be repaired at all. Therefore, it would be of interest to estimate quantitatively the probability of recovery per time unit and the fraction of irreversible damage for various combinations.

It is known (Kapultsevich, 1978; Korogodin, 1993) that the decrease in the effective dose $D_{eff(t)}$ with the recovery time t was fitted to an equation of the form:

$$D_{eff(t)} = D_1[K + (1 - K)\, e^{-\beta t}], \qquad (1)$$

where D_1 is the initial radiation dose; K is an irreversible component of radiation damage; e is the basis of the natural logarithm, and β is the recovery constant characterizing the probability of recovery per time unit. The estimation of the recovery parameters (K and β) after different combined treatments, at which a considerable synergistic interaction occurred make it possible to determine whether the mechanism of synergistic interaction was related to the impairment of the recovery capacity *per se* or to the production of irreversible damages. For various biological objects and different conditions of the combined action, the irreversible component increased with either the exposure temperature or the concentration of chemicals while the probability of recovery stayed unchanged. It can be concluded on this basis that the recovery process itself is not damaged and the inhibition of recovery is entirely due to the enhanced yield of irreversibly damaged cells.

4. MATHEMATICAL MODEL OF SYNERGISTIC INTERACTION

The results of the previous section, pointing out the negligible role of the recovery inhibition itself in the mechanism of synergistic interaction, strongly invoke

the need to elaborate a new theoretical conception of the synergy which, being useful for environmental radiation protection, take into account the regularities revealed. It might be reasonable to assume that some additional lethal lesions produced during combined action are responsible for the synergistic interaction. The supposition is that the additional lethal lesions are arisen from the interaction of sublesions induced by both agents and these sublesions are non-lethal when each agent is applied separately. It is assumed that one sublesion produced for instance by ionizing radiation interacts with one sublesion from another environmental agent (for specificity sake, let it be heat) to produce one additional lethal lesion. It would seem probable to suppose that the number of sublesions was directly proportional to the number of lethal lesions. Let p_1 and p_2 be the number of sublesions that occur for one lethal lesion induced by ionizing radiation and hyperthermia, respectively. Let N_1 and N_2 be the mean numbers of lethal lesions in a cell produced by these agents. A number of additional lesions N_3 arising from the interaction of ionizing radiation and hyperthermia sublesions may be written as

$$N_3 = min\{p_1N_1;\ p_2N_2\}. \qquad (2)$$

Here, $min\{p_1N_1;\ p_2N_2\}$ is a minimal value from two variable quantities: p_1N_1 and p_2N_2, which are the mean number of sublesions produced by ionizing radiation and hyperthermia, respectively. Thus, the model describes the mean yield of lethal lesions per cell as a function of ionizing radiation (N_1), hyperthermia (N_2), and interaction ($min\{p_1N1;\ p_2N_2\}$) lethal lesions. Then the synergistic enhancement ratio k may be expressed as

$$k = (N_1 + N_2 + N_3)/(N_1 + N_2). \qquad (3)$$

Taking into account Eqn. 2, the last expression can be rewritten as

$$k = 1 + min\{p_1;\ p_2N_2/N_1\}/(1 + N_2/N_1). \qquad (4)$$

It is evident from here that the highest synergistic interaction will be determined by the least value from the two functions: $f_1 = 1 + p_1/(1 + N_2/N_1)$ and $f_2 = 1 +$

$(p_2N_2/N_1)/(1 + N_2/N_1)$. Fig. 44A shows the dependence of both this functions on the ratio of N_2/N_1, calculated for arbitrary chosen p_1 and p_2 (p_1 = 6, p_2 = 4). The bold line at this figure depicts the dependence of the synergistic enhancement ratio on the ratio N_2/N_1, i.e. the ratio of the effects produced by each agent used in combination. Since f_1 decreases while f_2 increases with N_2/N_1, the greatest synergistic effect will be obtained when $f_1 = f_2$, i.e.

$$p_1/(1 + N_2/N_1) = (p_2 N_2/N_1)/(1 + N_2/N_1). \quad (5)$$

From here, the condition of the highest synergistic interaction can be obtained:

$$p_1N_1 = p_2N_2. \quad (6)$$

It means that the highest synergistic interaction occurred when both agents produce the equal number of sublesions. Taking into account Eqns. 3 and 5, the value of the greatest synergistic enhancement ratio is given by

$$k_{max} = 1 + [p_1p_2/(p_1 + p_2)]. \quad (7)$$

Some examples of theoretically predicted dependency of the synergistic enhancement ratio on the N_2/N_1 ratio for various values of the basic model parameters $p1$ and p_2 are depicted in Figs. 44B and 44C. If the observed biological effect is mainly induced by heat ($p_1N_1 < p_2N_2$) then taking into account Eqn. 4, the parameter p_1 can be expressed as

$$p_1 = (k1 - 1)(1 + N_2/N_1), \quad (8)$$

where k_1 is the value of synergistic enhancement ratio observed in experiments performed in this condition. On the contrary, if the observed biological effect is mainly induced by ionizing radiation, we have

$$p_2 = (k_2 - 1)(1 + N_1/N_2), \quad (9)$$

where k_2 is the experimental value of the synergistic enhancement ratio observed for the condition $p_2N_2 < p_1N_1$. The corresponding number of lethal lesions can be calculated (Haynes, 1966) as

$$N = - lnS, \qquad (10)$$

where S is the surviving fraction.

It is easily to demonstrate that the model under consideration can predict two N_2/N_1 ratio, at which equieffective values of the synergistic enhancement ratio (k_i) can be observed. For the case $p_1N_1 < p_2N_2$, we have

$$N_2/N_1 = (p_1 - ki + 1)/(ki - 1), \qquad (11)$$

while for the case $p_2N_2 < p_1N_1$

$$N_2/N_1 = (k_i - 1)/(p_2 - k_i + 1). \qquad (12)$$

Some examples of calculations based on Eqns. 11 and 12 can be found elsewhere (Petin *et al.*, 2000).

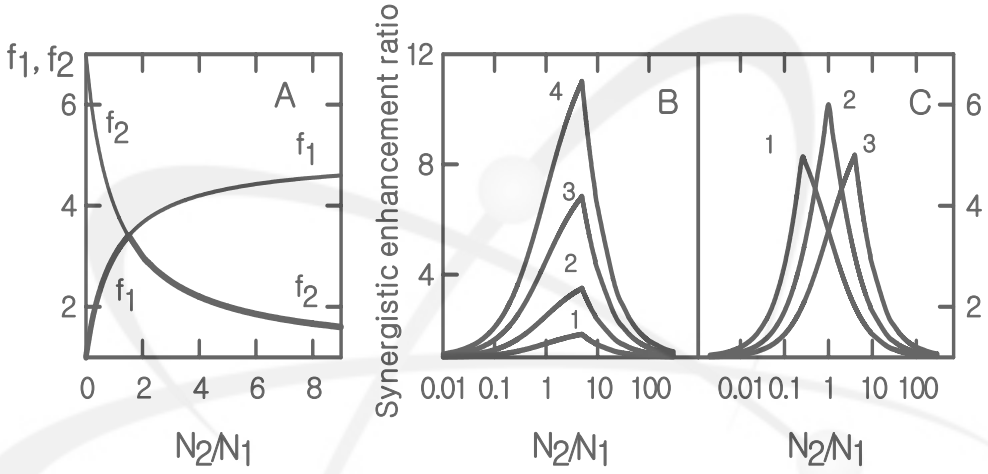

Fig. 44. The calculated dependencies of functions f_1 and f_2 on the N_2/N_1 ratio for the following values of the basic parameter: p_1 = 6 and p_2 = 4 (A) and theoretically expected dependencies of the synergistic enhancement ratio on the N_2/N_1 ratio for the following values of the basic parameter: B – p_1 = 5, p_2 = 1 (curve 1), p_1 = 15, p_2 = 3 (curve 2), p_1 = 35, p_2 = 7 (curve 3), p_1 = 60, p_2 = 12 (curve 4); C – p_1 = 5, p_2 = 20 (curve 1), p_1 = 10, p_2 = 10 (curve 2), p_1 = 20, p_2 = 5 (curve 3).

5. COMPARISON OF THE MODEL PREDICTIONS AND EXPERIMENTAL DATA

Several examples of this model application for optimization and prediction of the synergy have already been published (Petin and Komarov, 1997; Petin *et al.*, 1999; Petin *et al.*, 2000). The main value of the mathematical approach presented is the possibility to predict the equieffective synergy including the highest synergism and the N_2/N_1 ratio at which it can be achieved. Tests have been done on the applicability of the model for quantitative description, prediction and optimization of the synergistic interaction observed for various biological objects and test systems.

Fig. 45 presents the experimentally obtained (circles) and theoretically predicted (solid lines) relationships between the synergistic enhancement ratio and the N_2/N_1 ratio for inactivation of T_4 bacteriophage (A), *Bacillus subtilis* spores (B), diploid *Saccharomyces cerevisiae* yeast cells (C) and cultured mammalian cells (D). The procedures for calculating these relationships have been described in detail in the previous section. Initial experimental data used for these calculations were taken from earlier papers on simultaneous thermoradiation inactivation of bacteriophage (Trujillo and Dugan, 1972), bacterial spores (Raynold and Garst, 1970), yeast (Petin and Komarov, 1997) and mammalian cells Ben-Hur *et al.*, 1974; Ben-Hur, 1976). The errors in the synergistic enhancement ratio values (k) were calculated, if it was possible, from interexperimental variation. Predicted values of k were estimated by Eqn. 3 using the basic parameters p_1 and p_2 of the model which have been derived (Eqns. 7 and 8) from real experiments.

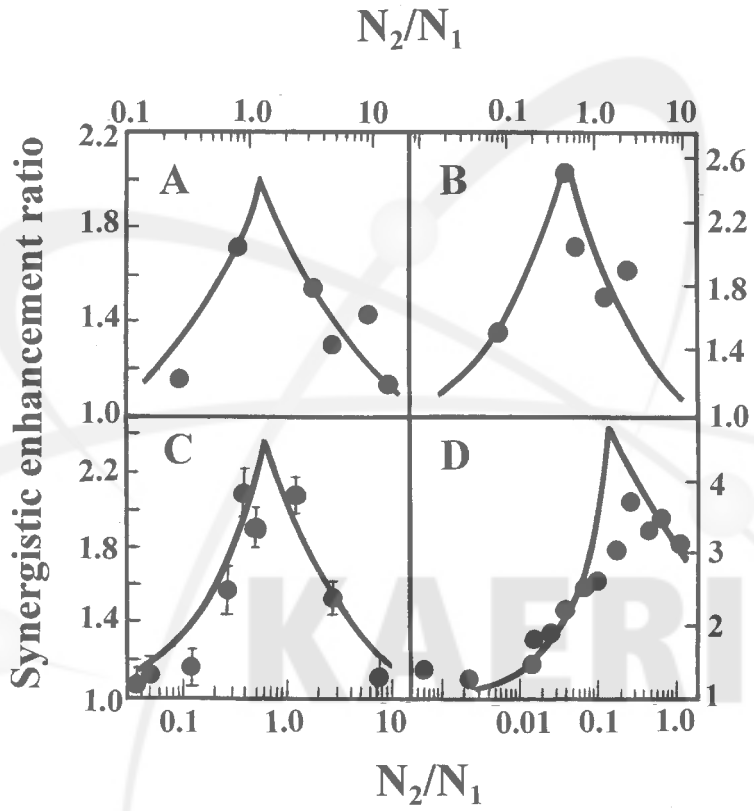

Fig. 45. Experimentally obtained (circles) and theoretically predicted (solid lines) dependencies of the synergistic enhancement ratio k on the N_2/N_1 ratio for simultaneous thermoradiation inactivation of T4 bacteriophage (A), *Bacillus subtilis* spores (B), diploid *Saccharomyces cerevisiae* yeast cells (C) and cultured mammalian cells (D).

6. CONCLUSIONS

The main common regularities of the synergistic interaction obtained in this investigation may be summarized as follows. (1) For any constant rate of exposure, the synergy can be observed only within a certain temperature range. (2) The temperature range which synergistically increases the effects of radiations is shifted to the lower temperature for thermosensitive objects. (3) Inside this range, there is a specific temperature that maximizes the synergistic effect. (4) A decrease in the exposure rate results in a decrease of this specific temperature to achieve the greatest synergy and vice versa. (5) For a constant temperature at which the irradiation occurs, synergy can be observed within a certain dose rate range. (6) Inside this range an optimal intensity of the physical agent may be indicated, which maximizes the synergy. (7) As the exposure temperature reduces, the optimal intensity decreases and vice versa. (8) The recovery rate after combined action is decelerated due to an increased number of irreversible damage. (9) The probability of recovery is independent of the exposure temperature for yeast cells irradiated with ionizing or UV radiation. (10) Chemical inhibitors of cell recovery act through the formation of irreversible damage but not *via* damaging the recovery process itself.

The remarkable result of this paper concerns the mathematical model that has been proposed to explain the experimental data of synergistic interaction of hyperthermia with other inactivating agents. The model is based on the supposition that synergism takes place due to the additional lethal lesions arising from the interaction of non-lethal sublesions induced by both agents. These sublesions are considered noneffective when each agent is applied separately. The idea of sublesions is widely used in radiobiology (Leenhouts and Chadwick, 1978; Murthy *et al.*, 1979; Zaider and Rossi, 1980). In the model, the synergistic effect is given by $min\{p_1N_1; p_2N_2\}$ (Eqn. 2). This means that one sublesion caused by irradiation or chemicals interacts with one sublesion produced by heat. This process is assumed to proceed until the sublesions of the less frequent type is used up. To estimate the basic parameters p_1 and p_2 we have used the experimental values of the synergistic enhancement ratio k_1 and k_2 (Eqns. 8 and 9). It means that the model takes into consideration only the actual interaction determining the synergistic effect. The model predicts the dependence of synergistic interaction on the ratio N_2/N_1 of lethal lesions

produced by every agent applied (Eqn. 4), the greatest value of the synergistic effect (Eqn. 7) as well as the conditions under which it can be achieved (Eqn. 6). The model is not concerned with the molecular nature of sublesions, and the mechanism of their interaction remains to be elucidated. In spite of the approximation used in this simplified model, it is evident from the data presented that a good agreement appears to exist between theoretical and experimental results. Moreover, the model discussed here can be used to predict conditions under which the greatest synergy can be observed and its value. The degree of synergistic interaction was found to be dependent on the ratio of lethal damage (N_2/N_1) induced by the two agents applied. The synergistic interaction is not observed at any N_2/N_1 ratios. The effectiveness of synergistic interaction appears to decline with a deviation of the ratio N_2/N_1 from optimal value.

The most interesting results, obtained from the model, is the conclusion that for a lower intensity of physical agents or a lower concentration of chemicals a lower temperature must be used to provide the greatest synergy. Actually, any decrease in the intensity of physical agents would result in a increase of the duration of thermoradiation action to achieve the same absorbed dose. Therefore, the number of thermal sublesions will also be increased resulting in the disruption of the condition at which the highest synergy should be observed (Eqn. 6). Hence, to preserve an optimal N_2/N_1 ratio with any decrease in the dose rate (or the intensity of other agents) the exposure temperature should be decreased. For a long duration of interaction, which are important for problems of radiation protection, low intensities of deleterious environmental factors may, in principle, synergistically interact with each other or with environmental heat.

제 3 장 결 론

제 3 장 결 론

“방사선융합 생물분자 구조변환 및 상승작용 이용 연구” 과제의 수행을 통하여 국가 방사선 핵심연구에 필요한 기반기술을 확하고 기존의 기술을 개선·발전시키고자 하였으며 다음과 같은 주요 내용과 범위가 연구개발에 포함되었다.

○ 단백질 또는 펩티드의 기능적 특성 증진을 위한 방사선 구조변환 기술 개발
○ 다당류의 물성개선 및 저분자화를 위한 방사선 구조변환 기술 개발
○ 생물유래 복합추출물 및 이차대사산물의 이화학적 특성 및 기능성 증진 기술 개발
○ 방사선 준위 및 복합처리 조건에 따른 생물영향 특성 연구
○ 방사선과 다요인 복합작용의 규칙성 도출 및 상승작용 이론모델 구축

방사선 융·복합 기술은 단순하게 방사선을 조사하여 그 영향 및 결과를 이용하던 단편적 기술의 한계를 극복하고 새로운 기술로 도약할 수 있는 다제간 신기술로서 다양한 이용범위를 가지며 산업적 파급효과가 높은 동시에 경제적 효과가 큰 강점 기술이다.

이온화 방사선의 에너지에 따라 방사선에 노출된 생물 개체 및 생체 물질 분자에서 나타나는 반응은 다양하다. 생물소재가 가지고 있는 고유 특성과 방사선 기술 및 생명공학기술을 복합 적용하여 신개념의 기능성 물질을 획득하는 등 괄목할 만한 연구성과를 이룩하였다. 연구 성과는 국제적 명성의 저명학술지에 논문으로 게재하고, 산업적 적용성이 입증도니 결과는 국내 및 국제 특허로 등록하는 등 연구 성과의 우수성과 연구수행의 성실성을 입증하였다. 연구결과는 후속 연구를 통해 더욱 개선되고 발전될 것이며 향후 새로운 연구과제 도출 및 수행을 위한 동기부여는 물론 기반기술로 제공될 것으로 전망된다.

방사선융합 기술을 적용하여 생체분자의 구조 및 기능을 변화시키고 이로부터 고부가가치 물질을 획득한 연구경험을 바탕으로 다양한 천연소재 및 농생물 소재를 이용하여 신물질을 개발하여 산업화하기 위한노력은 장기적 관점에서 볼 때 국가발전의 성장동력으로서의 의미를 가진다고 판단된다. 이온화 방사선과 물리적, 화학적 요인을 동시처리하거나 순차적으로 처리하는 복합처리 기법을 이용할 경우 방사선의 긍정적 이용효율의 극대화가 가능할 것으로 평가된다. 본 연구를 통해 다제한 연구와 기술융합을 촉진하는 계기가 마련될 것이다. 획득한 연구결과를 더욱 발전시키고 개선하여 방사선 이용기술의 고도화를 위한 연구개발 및 응용분야의 기반 을 확립함을 물론 기초과학, 산업, 의학 등 다양한 분야에 방사선을 효과적으로 활용하기 위한 기술근거 제공 효과를 거둘 수 있다.

KAERI

제4장 참고문헌

제 4 장 참고문헌

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SECTION 2 :

Improvement of the Physicochemical and Immunomodulatory Property of β-Glucan by Gamma-Irradiation

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SECTION 3 :

Improvement of Color and Antioxidant Properties of Chaga Mushroom (*Inonotus obliquus*) Extract by Gamma-Irradiation

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연구위탁기관			계약 번호		

초록(300단어 내외)

국가 방사선 핵심연구기관으로서 방사선 관련 연구개발의 기초기반 기술을 제공함으로써 새로운 연구과제 도출 및 수행을 위한 동기를 부여할 수 있다. 지금까지 방사선을 단순 조사 위주로 이용해 오던 평면적 기술에서 탈피하여 방사선 기술을 기반으로 기존 기술을 복합적으로 활용하여 한 차원 높은 방사선 융·복합 기술을 개발할 필요가 있다. 본 연구는 방사선 융합 기술을 이용하여 생물분자의 과학적, 산업적 이용가치를 창출하는 한편, 방사선의 긍정적 이용 효율을 극대화하기 위한 체계 정립에 역점을 두고 수행되었다. 방사선과 기존 기술을 병합한 RFT 기술의 적용을 통하여 생물분자의 구조를 변환함으로써 물리적, 화학적, 생물학적 기능이 증진된 물질을 확보하였다. 방사선과 기타 요인의 복합 처리 조건별 생물영향 평가 기술을 수립하고 이온화 방사선과 물리·화학적 요인을 복합처리할 때 나타나는 상승작용을 해석하고 최대 상승작용 조건 등을 사전 예측하는 이론을 정립함으로써 방사선의 긍정적 이용 효율을 극대화하기 위한 기술기반을 확립하였다.

주제명키워드 (10단어내외)

방사선, 분자구조변환, 복합처리, 생물영향

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Abstract(about 300 words)

It is expected that motivation and basic technologies for the future R&D plans can be provided from the results of this study. This study has been done to develop fundamentals for radiation applications based on the existing radiation technology, and to establish technical basis for enhancing efficacy of radiation utilization by studying the simultaneous application of ionizing radiation with another factor. Application of radiation technology together with the existing technologies to enhance the physical, chemical, biological characteristics through structural changes of biomolecules will exert a favorable influence on the creation of *de novo* scientific and industrial values. A theoretical model for the combined action of ionizing radiation with another factor can make it possible to predict *a prior* the maximum value of synergistic interaction and the conditions for it. Furthermore, the results of this study give a clues for establishment of fundamental theories associated with positive efficacy of radiation applications.

Subject Keywords (about 10 words)

biological effect, synergism, protein, polysaccharide, secondary metabolite