▣ PAPER ▣

RADIOPROTECTIVE EFFECT OF ALGIN-OLIGOSACCHARIDE THROUGH MEASURING CASPASE-3 AND CASPASE-9 IN MICE

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In order to find out the Radioprotective effect of algin-oligosaccharide(AOS), this study, with a mouse of which whole frame irradiated by 3 Gy radiation once, measured caspase-3 and caspase-9 amid cell signaling connected to apoptosis in order to observe cell activation.

In Caspase-3 and Caspase-9 test for observing cell activation, both of Caspase-3 and Caspase-9 showed highly increased O.D. value in the irradiation control group, while the whole groups treated with algin-oligosaccharide before or after irradiation indicated lower O.D. value than the irradiation control group, especially showed big difference in 7 day's treatment group of before irradiation ($P<0.001$). It confirmed that Caspase generation was restrained in AOS treatment group.

Consequently, this study inquired into the fact that algin-oligosaccharide with superior antioxidant activity performed radiation protection by inducing restraint of Caspase generation and confirmed that natural product with less chemical toxicity was able to be applied as radioprotector.

Keywords : Algin-oligosaccharide, Radioprotective Effect, Caspase-3, Caspase-9

1. INTRODUCTION

In recent academic world, as chemical protective materials to reduce radiation damage, Macro Glucan, TMG(vitamin E derivatives), Guarana, propolis, EEM (extracts of edible mushrooms), green tea, thio reagents, melatonin, vitamin C, and ginseng have been studied. However, most chemical protective materials have limits in use due to great toxicity involved in their effective dose, and particularly have a defect that should be treated prior to irradiation[1]. Thus recently, researches on biological response changes against radiation of some natural biological materials such as EGCG (epigallocathechin gallote), which is a major catechin component, have become a target for interest.

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Alginic acid is a component comprising cell wall in brown algae, such as brown seaweeds and tangle weeds and called also seaweed acid. Algin-oligosaccharide derived from alginic acid is known to accomplish a biological protective function through the mechanism eliminating free radicals or active oxygen by its excellent antioxydative effect[2]. Particularly, this mechanism prevents free radicals, active ions, or excited molecules to be expressed in biological macromolecule (e.g. DNA) by eliminating ions or excited molecules generated rapidly from physical and chemical actions following irradiation.

Caspase plays a role to induce apoptosis of seriously damaged cells by breaking down and eliminating apoptosis inhibiting proteins in cell nucleus progressing to apoptosis and is a group of cysteine protease to induce apoptosis only in cancer calls without any effect on normal cells in particular[3]. While the increase of caspase generation generally means that there is certain damage in the cell nucleus, the decrease of caspase generation means that the damage of cell is protected by

any other cause.

This study would explore How strong the protective effect of algin-oligosaccharide(AOS) was, by observing whether Caspase-3 and Caspase-9 were generated in irradiated mice with algin-oligosaccharide application. For this purpose, the mice were exposed to whole body radiations of 3 Gy at 1 time and applied with algin-oligosaccharide, and then Caspase-3 and Caspase-9 were measured following solubilization of intestine and liver tissues.

2. MATERIALS AND METHODS

2.1 Experimental Materials

2.1.1 Experimental Animals

Total 42 mice involving 7 mice a group of C57BL/6 mice (body weight: 25~35 g), which were produced and supplied by *Damul Laboratory Animal Center* (Korea) were used in this study and the intestine and liver tissues separated from the experimental mice were used for actual measuring. The mice were bred in cages made of polycarbonate(40 × 25 × 17cm) in a condition controlled animal room at temperature of 23±2°C and humidity of 45±5% and allowed free access to food (*CJ*, Korea) and tap water.

The experimental animals were used in the purpose to increase and protect human health in accordance with the idea of *Helsinki Declaration*, appropriate measures to minimize unfriendly effects on environment were taken, and we did every effort to establish an optimal well-being environment for the experimental animals during the whole process period of experiment.

2.1.2 Reagents

In this study, reagents such as Bicincchoninic acid protein assay kit (Pierce U.S.A), Tris (Bio Basic, USA), Ethylene diaminetetraacetic acid (EDTA, Sigma, USA), sodium chloride (NaCl, Junsei, Japan), Triton-X 100 (Junsei, Japan), sodiumnitrate (Sigma, USA), and Caspase-3/CPP32 & Caspase-9/Mch6 colorimetric assay kit (Biovision, USA) were used and specially algin-oligosaccharide(Sigma, USA), which is imported and supplied by EcoBio co., Ltd, Korea, was used.

2.1.3 Irradiation Equipment

For irradiation, Linac of 10 MeV grade (Clinac 21Ex, Varian, 2004) was used.

2.2 Classification of Experiment Groups

Experimental groups were established broadly as normal group, irradiation control group, treatment group of before irradiation, and treatment group of after irradiation, then the above treatment group of before irradiation was divided to AOS treatment for 7 days + irradiation group and AOS treatment for 3 days + irradiation group, and the above treatment group of after irradiation was divided to irradiation + AOS treatment for 3 days group and irradiation + AOS treatment for 7 days group(Table 1).

Table 1. Classification of experimental groups

	Experimental groups
Group 1	normal
Group 2	irradiation control
Group 3	AOS for 7days + irradiation
Group 4	AOS for 3days + irradiation
Group 5	irradiation + AOS for 3days
Group 6	irradiation + AOS for 7days

2.3 Experimental Methods

2.3.1 Irradiation

To test the protective effect of AOS against radiation damages, the mice received whole body irradiation of 300 cGy/min at 1 time, which was applied as 3 Gy in which range the biochemical changes in biological tissue might be expressed the most actively, by means of Linac radiation therapeutic equipment.

2.3.2 Administration of Reagents

Algin-oligosaccharide, as a major experimental material in this study, were applied in oral injection manner using oral injector under the standard of 5 mg/kg/day. The AOS was applied for 7 days prior to irradiation in AOS treatment for 7 days + irradiation group, for 3 days prior to irradiation in AOS treatment for 3 days + irradiation group, 3 days after irradiation in irradiation + AOS treatment for 3 days group, and for 7 days after irradiation in irradiation + AOS treatment for 7 days group.

2.3.3 Sacrifice of Experimental Animals and Collecting Experimental Tissues

In case of irradiation control group and treatment group before irradiation, the experimental animals were sacrificed simultaneously after 3 days from irradiation date with normal group, and in case of treatment group after irradiation, the mice were sacrificed after 3 days from the last treatment date of AOS. The experimental tissues were collected from the intestine and liver of mice immediately after sacrifice in all case. Cervical dislocation method was used for sacrifice.

2.3.4 Measuring Caspase-3 & Caspase-9

100 μg of each prepared intestine and liver tissues was put into each tube containing 100 μl of cell lysis

buffer and incubated in ice for 10 min for cell lysis. Each tube containing sample was centrifuged at 10,000 g for 1 min, then the supernatant of each tube was transferred to new tube and stored in ice. The protein quantification of each sample was conducted, then diluted to the concentration of 100~200 μg protein/50 μl cell lysis buffer, and each diluted sample was transferred to the prepared 96 well plate. After that, 50 μl of 2X Reaction buffer(reaction reagent, 10mM DTT contained) was added to each well, then 5 μl of 4 mM LEHD-*p*NA substrate was treated to each well, and the plate was incubated at 37°C for 1~2 hours. After incubation, the plate was measured using caspase-3/CPP32 & caspase-9/Mch6 colorimetric assay kit in a micro plate reader(micro-reader: 405 nm, USA).

2.4 Statistical Analysis

Each experimental result was expressed with mean and standard deviation(Mean±S.D.), ANOVA through SPSS 10.1 statistical program was performed for the significance evaluation among the experimental groups, and Tukey test was conducted as a post hoc test for multiple comparison among the experimental groups. The statistical evaluation was processed at the level of significance, $\alpha = 0.05$.

3. RESULT

3.1 Caspase-3

3.1.1 Small Intestine Tissues

In small intestine tissues from irradiated mice with algin-oligosaccharide treatment, caspase-3 was observed. The O.D. values at 405 nm resulted in 0.31±0.006 in AOS for 7days + irradiation group, 0.64±0.016 in AOS for 3days + irradiation group, 0.64±0.013 in irradiation + AOS for 3days group, and 0.62±0.011 in irradiation + AOS for 7days group and showed very significant differences($P<0.001$) of mean values compared with 0.72±0.045 in irradiation control group, and specially the largest difference appeared in AOS for 7days + irradiation group(Table 2, Fig. 1).

Table 2. Caspase-3 in small intestine of 3Gy irradiated mice with algin-oligosaccharide treatment

(O.D. : 405 nm)

Groups	Caspase-3 (mean±S.D.)
normal	0.02±0.010
irradiation control	0.72±0.045
AOS for 7days + irradiation	0.31±0.006***
AOS for 3days + irradiation	0.64±0.016***
irradiation + AOS for 3days	0.64±0.013***
irradiation + AOS for 7days	0.62±0.011***

***p<0.001 as compared with irradiation control group.

3.1.2 Liver Tissues

In liver tissues from irradiated mice with algin-oligosaccharide treatment, caspase-3 was observed. The O.D. values at 405 nm of in AOS for 7days + irradiation group resulted in 0.40±0.034 and showed very significant differences of mean values compared with 0.67±0.046in irradiation control group($P<0.001$)(Table 3, Fig. 1).

Table 3. Caspase-9 in liver of 3Gy irradiated mice with algin-oligosaccharide treatment

(O.D. : 405 nm)

Groups	Caspase-3 (mean±S.D.)
normal	0.20±0.060
irradiation control	0.67±0.046
AOS for 7days + irradiation	0.40±0.034***
AOS for 3days + irradiation	0.66±0.018***
irradiation + AOS for 3days	0.75±0.113***
irradiation + AOS for 7days	0.77±0.062***

***p<0.001 as compared with irradiation control group.

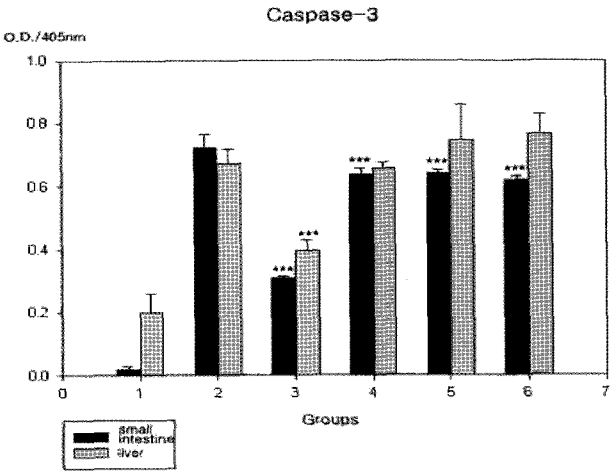

Fig. 1. Caspase-3 in small intestine and liver of 3Gy irradiated mice with algin-oligosaccharide treatment. The O.D values were increased in irradiation control group, the O.D values of all experimental groups treated with AOS before or after irradiation were lower than that of the irradiation control group, and particularly the value difference in AOS treatment for 7 days + irradiation group appeared very largely.
***p<0.001 as compared with irradiation control group.
Group 1 : normal
Group 2 : irradiation control
Group 3 : AOS for 7days + irradiation
Group 4 : AOS for 3days + irradiation
Group 5 : irradiation + AOS for 3days
Group 6 : irradiation + AOS for 7days

3.2 Caspase-9

3.2.1 Small Intestine Tissues

In small intestine tissues from irradiated mice with algin-oligosaccharide treatment, caspase-9 was observed. The O.D. values at 405 nm resulted in 0.30±0.029 in AOS for 7days + irradiation group, 0.61±0.010 in AOS for 3days + irradiation group, 0.64±0.006 in irradiation + AOS for 3days group, and 0.66±0.029 in irradiation + AOS for 7days group and showed very significant differences($P<0.001$) of mean values compared with 0.72±0.024 in irradiation control group, and specially the largest difference appeared in AOS for 7days + irradiation group(Table 4, Fig. 2).

Table 4. Caspase-9 in small intestine of 3Gy irradiated mice with algin-oligosaccharide treatment

(O.D. : 405 nm)

Groups	Caspase-3 (mean±S.D.)
normal	0.09±0.005
irradiation control	0.72±0.024
AOS for 7days + irradiation	0.30±0.029***
AOS for 3days + irradiation	0.61±0.010***
irradiation + AOS for 3days	0.64±0.006***
irradiation + AOS for 7days	0.66±0.029***

***p<0.001 as compared with irradiation control group.

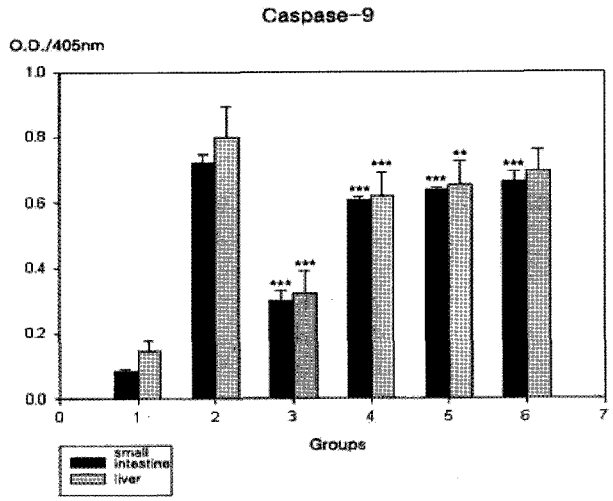

Fig. 2. Caspase-9 in small intestine and liver of 3Gy irradiated mice with algin-oligosaccharide treatment. The O.D values were increased in irradiation control group, the O.D values of all experimental groups treated with AOS before or after irradiation were lower than that of the irradiation control group, and particularly the value difference in AOS treatment for 7 days + irradiation group appeared very largely.
***p<0.001 as compared with irradiation control group.
**p<0.01 as compared with irradiation control group.
Group 1 : normal
Group 2 : irradiation control
Group 3 : AOS for 7days + irradiation
Group 4 : AOS for 3days + irradiation
Group 5 : irradiation + AOS for 3days
Group 6 : irradiation + AOS for 7days

3.2.2 Liver Tissues

In liver tissues from irradiated mice with algin-oligosaccharide treatment, caspase-9 was observed. The O.D. values at 405 nm resulted in 0.32±0.068 in AOS for 7days + irradiation group and 0.62±0.073 in AOS for 3days + irradiation group and showed very significant differences of mean values($P<0.001$) compared with 0.80±0.092 in irradiation control group, and particularly the largest difference appeared in AOS for 7days + irradiation group(Table 5, Fig. 2).

Table 5. Caspase-9 in liver of 3Gy irradiated mice with algin-oligosaccharide treatment

(O.D. : 405 nm)

Groups	Caspase-3 (mean±S.D.)
normal	0.15±0.029
irradiation control	0.80±0.092
AOS for 7days + irradiation	0.32±0.068***
AOS for 3days + irradiation	0.62±0.073***
irradiation + AOS for 3days	0.65±0.072**
irradiation + AOS for 7days	0.69±0.067

***p<0.001 as compared with irradiation control group.

3.3 Measurement of Radiation-Induced TNF-α

In measurement of radiation-induced TNF-α, small intestine and liver tissue indicated highly increased TNF-α value in the irradiation control group, while small intestine tissue showed restrained generation of TNF-α in 7 day's treatment group of before irradiation ($P<0.001$), and liver tissue showed suppressed creation of TNF-α in 7 day's treatment group of before irradiation ($P<0.001$), 3 day's treatment group of before irradiation,

Table 6. TNF-α in small intestine and liver tissue of 3Gy irradiated mice with algin-oligosaccharide treatment.

(unit : pg/mG)

Groups	Mean ± S.D.	
	small intestine	liver
normal	0.05±0.014	0.02±0.008
irradiation control	0.45±0.108	0.33±0.046
AOS for 7days + irradiation	0.29±0.052***	0.21±0.020***
AOS for 3days + irradiation	0.45±0.040	0.25±0.025***
irradiation + AOS for 3days	0.43±0.035	0.27±0.016**
irradiation + AOS for 7days	0.51±0.046	0.26±0.009***

***p<0.001 as compared with irradiation control group.
**p<0.01 as compared with irradiation control group.

and 7 day's treatment group of after irradiation. The finding confirmed TNF-α generation was restrained in AOS treatment group(Table 6).

4. DISCUSSION

Algin-oligosaccharide has been known to have a mechanism eliminating active oxygen and free radials generated from various stimulations through its antioxydative function and may be an effective protective measure of living body by eliminating rapidly the malignant neo-materials generated from ionizing and exciting reaction immediately after irradiation in case that a proper treatment is applied before or after irradiation.

In relation to the cell signaling pathway followed by cell death after irradiation, Wang *et al* (2002) reported that NF-kB, which was a major signal transfer material on developmental process of inflammation response, was an essential element leading cell death or cell survival in the transduction pathway signaling triggered by ionized radiation[4]. Zaidi *et al*(2004) reported that cell death induced from whole body irradiation might be reduced considerably, in case to be treated with light whole body thermotherapy before 20 hours of whole body irradiation of 8 Gy[5].

Hong *et al*(1997) reported through brain tissue responding against irradiation that c-fos and junB, rapid expressing proteins of brain nerve cells, reacted[6]. In addition, Claudia *et al*(1998) reported that enzymes such as stress responding protein kinase or protein kinase C known as a radiation hazard inducing factor, led to attenuation of cell division during G_1 period through inducing rapid reactive genes[7].

Apoptosis is an active process requiring energy, induced by various cell stimulations such as UV[8], ligand binding to the corresponding receptor[9], oxygen radical metabolite[10], and DNA damage from ionized radiation[11], and led to bubble generation in cell membrane caused by cleavage of fordrin and followed by apoptosis[12][13]. It has been reported that induction or inhibition of artificial programmed cell death may not only be possible and but also find drugs and physical methods allowing inhibition of programmed cell death [14][15].

In this study, caspase-3 and caspase-9 in cell signaling pathway leading to cell death after irradiation were observed. While about both caspase-3 and caspase-9, the OD values of irradiation control group displayed high increase, the OD values of all experimental groups treated with AOS before or after irradiation appeared significantly lower than that of irradiation control group. Moreover, the largest difference appeared in AOS treatment group for 7 days before irradiation in particular. Caspase play a role to induce apoptosis of seriously damaged cell by degrading and eliminating apoptosis inhibiting proteins in nucleus. It is considered that the protection against radiation hazard by antioxydative reaction of AOS results in down control of caspase OD value in experimental groups treated with AOS.

5. CONCLUSION

To explore the radiation protective effect of algin-oligosaccharide, caspase-3 and caspase-9 were measured in total 42 mice received whole body irradiation with 3 Gy radiation at 1 time, which comprised 7 mice of normal group, 7 mice of irradiation control group, 7 mice of AOS treatment group for 7 days before irradiation, 7 mice of AOS treatment group for 3 days before irradiation, 7 mice of AOS treatment group for 3 days after irradiation, and 7 mice of AOS treatment group for 7 days after irradiation.

While about both caspase-3 and caspase-9, the OD values were increased in irradiation control group, the OD values of all experimental groups treated with AOS before or after irradiation were lower than that of the irradiation control group, and particularly the value difference in AOS treatment group for 7 days before irradiation appeared very largely($P<0.001$). With this, we confirmed that the generation of caspase was inhibited in AOS treated group.

In conclusion, we identified that AOS with excellent antioxydative effect accomplished the radiation protective action by inducing the inhibition of caspase-3 and caspase-9 generation and confirmed that nature oriented materials with little chemical toxicity might be utilized as a radiation protective agent.

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