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원 저

글라이포세이트의 유전자 독성에 대한 멜라토닌의 유전자 보호 효과

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Genoprotective Effect of Melatonin Against to the Genotoxicity of Glyphosate on Human Blood Lymphocytes

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Purpose: Glyphosate is a widely used non-selective herbicide. Previous studies have shown that glyphosate has genotoxicity, and that even low-doses of glyphosate can cause DNA damage. Melatonin is a hormone produced and secreted by the pineal gland that is known to be a potent anti-carcinogen, anti-oxidant, and genetic protector. This study was conducted to investigate the genoprotective effect of melatonin against glyphosate in human blood lymphocytes.

Methods: Human peripheral blood was obtained from 15 young, healthy volunteers and cultured under four different toxicologic conditions. The four groups consisted of a control group, glyphosate only group (300 ng/mL), glyphosate with low level of melatonin group (50 μM), and glyphosate with high level of melatonin group (200 μM). The mean Sister Chromatid Exchange (SCE) frequency of each group was then analyzed.

Results: Glyphosate exposed groups had a higher mean SCE frequency (10.33±2.50) than the control group (6.78 ±2.31, p<0.001). Interestingly, the group that received a low-level of melatonin had a lower mean SCE frequency (8.67±2.58) than the glyphosate-only group, while the group that received a high level of melatonin had a much lower mean SCE frequency (8.06±2.50) than the glyphosate-only group. There was statistical significance.

Conclusion: Melatonin exerted a potent gene protective effect against the genotoxicity of glyphosate on human blood lymphocytes in a dose-dependent fashion.

Key Words: Glyphosate, Genotoxic, Sister chromatid exchange, Melatonin

Introduction

Glyphosate is widely used nonselective herbicide for both agricultural and non-agricultural purpose. The chemical name of glyphosate is 'N-(Phosphonomethyl) glycine'. It was discovered to be an herbicide by Monsanto chemist John Franz in 1970. Many previous studies about the safety of glyphosate formulation have concluded that there is little toxicity to humans. Past review concluded that there is no

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potential for Roundup® (the registered trademark of Monsanto, One of the most widely used glyphosate commercial formulation) herbicide to pose a health risk to humans[1]. According to more extensive use of glyphosate, glyphosate intoxicated patients visited emergency room more frequently. However, recent studies showed a harmful effect of glyphosate variously. Especially, their harmful effect is vary depending upon absorption route. Small amount of oral intake may cause nausea, vomiting, and diarrhea, however, large amount of ingestion may cause severe systemic effects. Severe systemic symptoms may occur from cardiotoxicity, hepatotoxicity, renal toxicity, non-cardiogenic pulmonary edema, mental change, metabolic acidosis and even to cardiac arrest and death. And inhalation exposure may cause airway discomfort, laryngeal burn, and acute pneumonitis[2]. But most of those symptoms are acute clinical chief complaints. Glyphosate has much toxicity in addition to acute clinical chief complaints. Glyphosate herbicide is potential endocrine disruptor to human. And cytotoxicity of glyphosate herbicide on placental cells could induce reproductive disability[3]. Moreover, glyphosate herbicide induces the breakage of DNA strands and chromosomal damage[4]. Previous study showed that glyphosate has the genotoxicity by sister chromatid exchange (SCE) test, and even a low-dose of glyphosate may cause DNA damage[5]. Several studies about glyphosate supported that glyphosate has a negative effect for human[6-8]. Glyphosate may have various toxicities but antidote does not exist. So, conservative management is almost only treatment in acute intoxicated patient. And there are no preventable agent for genotoxicity and carcinogenicity of glyphosate.

Melatonin, chemically N-acetyl-5-methoxytryptamine, is a manmade form of hormone produced and secreted by the brain, exactly pineal gland. Known effects of melatonin is regulating circadian rhythm of physiological functions including bedtime, seasonal reproduction, blood pressure, core temperature, and so on[9,10]. In addition to widely known effect above, melatonin has many beneficial effects physiologically. Melatonin increases activity of anti-oxidative enzymes and scavenges free radicals as an antioxidant[11,12]. And, melatonin has been known potent anti-carcinogen. Many studies were performed about anti-carcinogenicity of melatonin through various methods. These studies showed oncostatic action or even tumor size reduction effect of melatonin for breast cancer, colon cancer, cervicovaginal cancer, and hepatocellular cancer[13-17]. Randomized controlled trial demonstrated that melatonin reduced the risk of death at 1 year in solid cancer patients[18]. Melatonin is also potent genetic protector. Previous study proved that melatonin reduced DNA damage induced by gamma radiation[19]. And melatonin reduced genotoxic effects by hypoxia both in vitro and in vivo[20]. Anisimov et al.[21] showed that melatonin protected the cells from DNA damage, not only by oxidative mutagens, but also by different alkylating agents.

It is difficult to know the detail mechanisms of both genotoxic effect of glyphosate and genetic protective effect of melatonin. This study was done to clarify protective effect of melatonin in human blood lymphocyte exposed to genotoxicity of glyphosate by SCE frequency method.

Methods

1. Chemicals

Roundup UltraMax® (Monsanto, Roseville, CA, USA) was used as representative product of glyphosate herbicide. It contains 570 gram of active ingredient glyphosate in 1 liter. And Melatonin made by Sigma-Aldrich (Saint Louis, MO, USA) was used. Phytohemagglutinin (PHA, Gibco, Waltham, MA, USA) as mitogen, RPMI 1640 (Gibco, Waltham, MA, USA) as culture medium, fetal bovine serum (Gibco, Waltham, MA, USA) as growth supplement and colcemid (Gibco, Waltham, MA, USA) were used. Bromodeoxyuridine (BrdU, Sigma-Aldrich, Saint Louis, MO, USA) was also used.

2. Blood Sampling

Peripheral blood of young and healthy volunteers

aged from 20 to 24 years was collected because aging can affect SCE frequency[22]. This experiment was approved by the institutional review board (IRB), and all subjects of this experiment signed on an informed consent. To control the factors that can change SCE frequency, regular drug users, smokers and alcoholics were excluded. And anybody who had cancer or chronic infection or history of chemotherapy or history of radiotherapy or radiation exposure history was also excluded, too. Finally, 15 people were included (9 males and 6 females). From each subject, 4.0 mL of peripheral blood was sampled and heparinized.

3. Preparation and Treatment

All of blood samples were experimented together through four groups that divided by concentrations of glyphosate and melatonin. Group 1 is control group that contains no glyphosate and no melatonin. Group 2, 3, and 4 are experimental groups. Group 2 contains only 300 ng/mL of glyphosate and no melatonin, and Group 3 contains 300 ng/mL of glyphosate and 50 μM of melatonin, and Group 4 contains 300 ng/mL of glyphosate and 200 μM of melatonin.

One milliliter of blood from each sample was cultured in 9 mL of RPMI-1640 as culture medium and PHA (1 g/mL) was added as mitogen. After 24 hours of incubation, 0.1 mL of BrdU (1 g/mL) was added. Different concentrations of glyphosate and melatonin according to groups were added after 48 hours of incubation. At last, after 70 hours of incubation, 0.1 mL of colcemid (10 μg/mL) was added to induce mitosis arrest at metaphase. So, 72 hours of incubation period was finished. All of the samples were treated under the same condition except only concentration of glyphosate and melatonin.

4. Sister Chromatid Exchange (SCE) Assay

Lymphocytes in all groups were collected by centrifugation. And swollen in 75 mM of hypotonic potassium chloride (KCl) solution for 10 min, and used 3:1 methanol-acetic acid for fixation. Centrifuging, fixing, and re-suspending in fresh fixative was repeated three times. These cells were dropped onto the slides carefully. Cells on the slides were stained by Höechst-Giemsa method to examine SCE frequency. Cytovision Karyotyping System (Applied Imaging, San Jose, CA, USA) was used to analysis SCE frequency of lymphocytes. For each group in one subject, 20 of well-spread chromosome

Table 1. Mean SCE frequency of individual subject

Subjects	Sex	Groups			
		Control	GLY (+)	GLY (+) & MLT (+)	GLY (+) & MLT (++)
1	M	7.35	10.20	8.10	7.50
2	M	6.45	10.65	7.55	9.80
3	M	7.45	10.50	8.10	8.95
4	M	6.00	10.85	9.68	8.90
5	M	6.90	10.95	9.75	7.75
6	M	6.90	13.85	10.30	8.75
7	M	7.80	12.20	9.00	8.35
8	M	6.85	11.75	8.25	6.85
9	M	6.70	11.35	8.25	8.10
10	F	5.65	11.60	8.95	8.85
11	F	6.45	11.00	9.17	7.50
12	F	6.70	11.60	8.80	7.50
13	F	6.80	10.50	8.05	7.85
14	F	6.90	10.40	8.45	7.05
15	F	6.75	10.05	7.95	7.15

GLY: glyphosate, MLT: melatonin, SCE: sister chromatid exchange

pairs in second division metaphase were included in results. Totally, 1,200 of lymphocyte were examined and the mean SCE count was statistically analyzed.

5. Statistical Analysis

In this experiment, Student's t-test and Mann-Whitney methods were used to statistically compare the mean number of SCEs from each group. All statistical analyses were performed using SPSS for windows (ver 20.0 SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered significant.

Results

Mean frequency of SCEs (SCE count/cell) was examined and statistical analysis was performed. Average SCE frequency of each group in individual subject of this study was presented in Table 1. The mean and standard deviation (SD) of each group were calculated and summarized in Table 2. Exposure group to glyphosate (Groups 2, 3, and 4) has a higher mean SCE frequency than Group 1, significantly. Compared to mean SCE frequency of Group 1 (6.78±2.31), Group 2 (11.16±2.70) had extremely higher SCE ($p<0.001$).

And Group 3 and 4 revealed decrease in mean SCE frequency (8.67±2.58; 8.06±2.50, respectively) when compared with that of group 2 ($p<0.001$, $p<0.01$). Group 3 and group 4 also showed statistically different SCE frequency ($p<0.05$). These results indicated that melatonin reduced SCE frequency by glyphosate with dose dependent manner.

Different result between genders was presented on the Table 3. Mean SCE frequency of male was slightly higher than that of female, in all groups though they did not get statistical significance. In female, mean SCE frequency of group 4 showed lower value than that of Group 3 with statistical significance ($p<0.05$). In male, mean SCE frequency of group 4 showed also lower value than that of Group 3, same as in the female, however, there was no statistical significance.

Table 2. Effect of melatonin in genotoxic lymphocyte by glyphosate

Groups	Mean SCE frequency (Mean±SD)
Control	6.78±2.31
GLY (+)	11.16±2.70*
GLY (+) & MLT (+)	8.67±2.58*,†
GLY (+) & MLT (++)	8.06±2.50*,†,‡

GLY: glyphosate, MLT: melatonin, SCE: sister chromatid exchange
*: $p<0.001$ compared to control
†: $p<0.001$ compared to 'GLY (+)'
‡: $p<0.05$ compared to 'GLY (+) & MLT (+)'

Discussion

Purpose of this study was to know the genoprotective effect of melatonin against to the genotoxicity of glyphosate on human blood lymphocytes. Result showed that glyphosate causes DNA damage and genotoxicity that related to mutagenicity and carcinogenicity. Also, this study showed melatonin has genetic protective effect against genotoxicity of

Table 3. Gender-based different effect of melatonin in genotoxic lymphocyte by glyphosate

Groups	Mean SCE frequency (Mean±SD)	
	Male	Female
Control	6.93±2.37	6.54±2.19
GLY (+)	11.37±2.76*	10.86±2.58*
GLY (+) & MLT (+)	8.77±2.77*,†	8.52±2.24*,†
GLY (+) & MLT (++)	8.33±2.43*,†	7.65±2.54*,†,‡

GLY: glyphosate, MLT: melatonin, SCE: sister chromatid exchange
*: $p<0.001$ compared to control
†: $p<0.001$ compared to 'GLY (+)'
‡: $p<0.05$ compared to 'GLY (+) & MLT (+)'

glyphosate with dose-dependent manner for the first time. Prior to discussion of these results, it is necessary to know more detail about the SCE. Sister chromatid exchange (SCE) is the exchange of genetic material between two identical sister chromatids. SCE method in peripheral blood lymphocytes is well known technique for examine genotoxicity of agent[23]. After chromosomal double-strand breaks (DSBs), inter-strand cross-linking damage, and collapsed replication forks by DNA damage was occurred, homologous recombination (HR), the important pathway of genomic repair, is necessary for DNA repair. When demand of HR increased, available sequence from the sister chromatid is used and dysregulated HR may occur. The result of above mechanism is SCE, so increased SCE frequency means resultant of severe DNA damage[24,25]. Because there is no direct association between SCE and health outcome[26,27], it is controversial that usefulness of SCE frequency as an early biomarker for carcinogenicity of any organism. However it is widely accepted that SCE is closely related to genotoxicity, and closely related to carcinogenicity. In addition, one of recent study reviewed that SCE assay can be used as a predictor of tumor chemoresponse[28].

As result of this study, all of glyphosate exposed group including group 2 (11.16±2.70), group 3 (8.67 ±2.58), and group 4 (8.06±2.50) have a significantly higher value of mean SCE frequency than control group (6.78±2.31). This result supported that glyphosate has genotoxicity and this result and its degree were in agreement with previous SCE study[24-26].

Interestingly, melatonin contained group 3 and 4 have lower value of mean SCE frequency than group 2 (p<0.001). This result suggested that melatonin has protective effect to genotoxicity of glyphosate. Comparing between group 3 and group 4, higher dose of melatonin showed more powerful preventive effect on this genotoxicity, indicating its dose dependent effect.

When stratifying the subjects into gender, different feature of melatonin treatment was found. Significant difference of SCE frequency between Group 3 and Group 4 was shown only in female not in male. This result demonstrated that high dose of melatonin had more beneficial effect on glyphosate genotoxicity in female, however, it had little effect than low-dose of melatonin in male. This result was originated from some male subjects (No. 2 and 3) with higher SCE frequency in Group 4 than Group 3. Unexpectedly, high dose of melatonin showed an negative effect in some subjects, and this results was in agreement with previous result. Lee et al.[20] demonstrated that high dose of melatonin treatment occurred increased SCE frequency in vivo. However, its detail mechanism and signal pathway was not clarified in this study. Therefore, melatonin effect on the genotoxicity by glyphosate should be confirmed with larger samples further.

However, melatonin was still beneficial and attractive chemical considering its various effects. In vivo study of Galley et al.[29] showed melatonin had beneficial effects on sepsis by recover mitochondrial dysfunction, oxidative stress, and cytokine responses. And Shokouhi et al.[30] showed a neuro-protective effect of melatonin in peripheral neural fibers from lipid peroxidation damage after blunt trauma. Based on these achievements, immediate-release melatonin is widely available as a dietary supplement. However, its side effects like present study have not been recognized. Therefore, the best effective and safe dose of melatonin should be evaluated further.

This study has some limitation. At first, various concentration of glyphosate and melatonin were analyzed in preliminary study, however, only 3 groups were analyzed in main experiment. Moreover, the toxicity of pure glyphosate and Roundup® may be different. And second, this was performed as in vitro study, therefore, in vivo study should be needed to know the definite effect and clinical availability of melatonin for glyphosate exposed patient. And third, DNA damage was evaluated by only SCE analysis no other direct analysis.

Taken together, these results suggest that melatonin may be a potential supplement for the genotoxicity of glyphosate. Its regular treatment might be helpful to the individuals who exposed to glyphosate frequently or chronic glyphosate exposure. And, to

prevent glyphosate intoxication and to improve usage of melatonin, further evaluation was needed to know followings:

1. Would there be same result in situation of in vivo study?
2. What is safe and effective dose of melatonin?
3. Is there any other treatment agent for genotoxicity of glyphosate?
4. Is there any other beneficial effect of melatonin?

Conclusion

This study demonstrated that melatonin has genetic protective effect in human lymphocyte exposed to genotoxicity of glyphosate with dose-dependent manner. This study may contribute to safe and effective use of melatonin in the status of glyphosate exposure.

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