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THE ROLE OF SOY IN PREVENTING APOPTOSIS IN LIVER INJURY

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ABSTRACT

Where there is liver injury, by any cause, there are numerous apoptotic cells which influence the metabolic function of the liver. Soy isoflavone is known to have an effect that inhibits apoptotic cells in follicles and osteoblast. The aim of this study is to evaluate whether soy has an anti-apoptotic effect on CCl4induced liver injury in mice. This study used 30 eight- to 10-week-old male DDY mice, divided into six groups. Group I acted as positive control, and received standard pellets with injury induced by 0.2 ml CCl_4 per oral intake, for 3 weeks. Group II, the negative control, received only standard pellets. Groups III-VI received standard pellets treated soybean extract at 145.6 mg, 218.4 mg, 291.2 mg and 364 mg per day respectively, administrated orally for 3 weeks and then received0, 2 ml CCl_4 per oral intake for the inducement of injury. After 4 days ofCCl_4,-induced intake the effect of the soybean extract was evaluated using histochemistry evaluation TUNEL (Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling). The identification and quantification of the apoptotic cells in the mouse liver tissue were done using light microscopy, and the TUNEL immunohistochemical examination results showed that the number of cells undergoing apoptosis in the group treated by soybean extract was less than those in the group that was not treated. Enhancement by ANOVA analysis between the groups showed a significant difference at $p < 0.05$. The results showed that the soy treatment when administrated orally, could significantly prevent the development of apoptotic cells in liver injury.

KEYWORDS

Apoptotic, Liver Injury, CCl_4, Isoflavone, Soybean and TUNEL.

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INTRODUCTION

In both acute and chronic liver disease, there are numerous apoptotic processes, occurring in cases where liver disease is caused by viral or autoimmune hepatitis, cholestatic disease, liver disorders due to alcohol/drug toxicity and liver damage after transplantation, including ischemic reperfusion injury as a consequence of graft rejection. Almost all forms of disruption to the liver can lead to the

destruction of hepatocytes. Liver failure occurs when there is a decline in hepatocyte function which leads to the liver no longer being able to complete metabolic and synthesis functions[1-3]. Induction of liver injury by CCl4 administration resulted in changes similar to the changes that occur in liver tissue injury due to various abnormalities[4-7].

In a study undertaken in several previous investigations, it was found that there are a large number of hepatocytes undergoing apoptosis in acute liver injury due to CCl4 administration in mice. Apoptotic cells can be identified and counted using light microscopy or electron microscopy, in situ immunohistochemical labelling of nuclear DNA fragmentation, flow cytometry, and DNA gel electrophoresis[4-11].

Soybeans and soybean products are rich in phytoestrogen isoflavone such as genistein, daidzen, and glycitein. Results from research at the National Soybean Research Laboratory of The Soy/Swine Nutrition Research Programme,at the University of Illinois in 1999, show that daidzein and genistein are the types of phytoestrogens contained in soy which demonstrate the ability to reduce apoptosis. The capability of daidzein is shown to be ten times greater than that of genistein. Both can potentially improve the survival of follicles, which leads to an increase in follicle size[12-14].

Research conducted by Kwang Sik Suh *et al* in 2003 also concluded that soy isoflavones are proven to inhibit apoptosis in cells where osteoblast is present[15]. However to date there is no information regarding the effect of soy on liver cell apoptosis in liver injury, which can be used as an indicator of hepatocyte survival.

In this study, we administered soy extract to measure its effect on liver tissue damage due to CCl4 administration in laboratory mice. The parameters measured were the number of apoptotic cells, to assess the effect of soy on the prevention of apoptosis in liver tissue. The hypothesis was that in the treatment group where soy extract was administered, the number of apoptotic cells would be less than the group which had not been given soy extract.

MATERIAL AND METHODS

Subjects of Research

The subjects were 30 eight- to 10-week-old male DDY strain mice. The mice were allowed to adapt to the laboratory environment for seven days. Then the mice were divided into six treatment groups. Group I was given no soy extract, and only fed pellets of 4 g/day, given a drink of water and 0.2 ml of CCl4. Group II was given no soy extract, only fed pellets of 4 g/day, given a drink of water and not given CCl4. Groups III-IV were given soy extract orally at 145.6 mg/day, 218.4 mg/day, 291.2 mg/day and 364 mg/day respectively, in addition to being fed pellets 4 g /day, drinks of water and being given 0.2 ml CCl4.

After soy extract administration for three weeks, the mice were given 0.2 ml of CCl4 orally for four days. Samples were collected from the livers of the mice postmortem and TUNEL and immunohistochemical examinations of the liver tissue were done using a light microscope, to count the number of apoptotic cells.

Soybean Extraction

The soybeans were processed in the laboratory, and were first boiled for 15 minutes and then dried in a heating apparatus at 40°C for two days to reduce the water content of the composition without damaging the nutrients contained in them. Then the dried soybeans were ground to the consistency of flour. The soybean flour was then dissolved in water with a volume ratio of water to soybean flour adapted for each treatment group.

Minimum effective concentration of soy as a food supplement in humans is 0.8 g/kg/day, so the needs of an adult with a body weight of 70 kg is 0.8 x 70 = 56 g/day which is comparable to 0.05% of total daily calorific intake. For the purposes of this study, the ratio was converted for mice according to the Paget and Barnes conversion table. Thus, 0.0026 x 56 = 0.1456 g/day or 145.6 mg/day was established as the minimal effective concentration (Level 1). Level 2 was set as 1.5 x 145.6 mg = 218.4 mg/day, Level 3 as 2 x 145.6 mg = 291.2 mg /day and Level4as 2.5 x 145.6 mg = 364 mg/day.

Carbon tetra chloride

The CCl4 dose recommended by Djamal et al to affect changes at the tissue level is 0.2ml (equivalent to 16.47mg CCl4 in solution) to be administered orally[16].

TUNEL Immunohistochemical examination

The method to detect cells undergoing apoptosis can be based on measuring the characteristics of apoptosis; one of which is the occurrence of DNA fragmentation. A common method used to detect DNA fragmentation is enzymatically by using the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling). The TUNEL reagent consists of terminal transferase enzyme at a charge recognised at 3'OH ends (nick end) generated by DNA fragmentation and fluorescein-dUTP to make the 3'OH end visible, where results can be observed using fluorescent microscopy, flow cytometry or light microscopy.

This research used a light microscope to measure apoptotic cells in comparison with non-apoptotic cells invisual field observations, used the double-staining method with TUNEL reagent and Giemsa. TUNEL detects only apoptotic cells and produces a brown color, while Giemsa is used to detect non-apoptotic cells and provides a purplish-blue color.

The following is a description of the procedure undertaken. Firsta slide containing polylisine and a tissue incision was placed in a 56-60ºC oven for 15 minutes. Then deparafinizing preparations were made with xylene (3 times each for 3 minutes). Nextthe rehydration aspect was added, using 100% ethanol, 95% ethanol and 70% ethanol, as well as rehydration preparations with sterile distilled water, and endogenous POD inactivation by H2O2: water (aspect ratio 1:9) for 5-10 minutes. Next samples were washed in a PBS solution, with protease added before incubation for 30 minutes at 37°C where samples were covered with tin foil. Following incubation, samples were washed again with PBS and a permeabilising incision was made (with 2 minutes on ice), and 50 mL TUNEL labelling mix (consisting of 5 mL deoxynucleotydil terminal transferase enzyme and fluorescein-dUTP 45 mL) was used in drops, before incubation for 60 minutes at 37ºC. Following this incubation, samples had another PBS wash before adding anti-fluorescein-POD and incubation (this time for 30 minutes at 37°C). Another washPBS took place, and DAB substrate was added before a final incubation period of 5-20 minutes, RT. A final wash with PBS occurred and then samples were washed with water before providing a counter stain. Samples received a further wash with water and were patted dry, dipped into absolute alcohol to clear, and dried. Closing preparations were made with entelan and silicone cover slips. The samples were then observed and apoptotic cells (now brown) were counted using light microscopy.

Data analysis

Data analysis was performed using an ANOVA (Analysis of Variance) parametric test.

RESULTS AND DISCUSSION

Immunohistochemical examination of TUNEL

The results of the histopathologic observations from Groups III to VI, who were given soy extract with four levels of variation in administration at 145.6 mg/day, 218.4 mg/day, 291.2 m/day and 364 mg/day, and were administered0.2ml CCl4, display a microscopic picture similar to that of Group II, which was the normal liver tissue of mice without treatment (Figure No.1). In general, GroupsIII-VI, where observed with a magnification of 40X, showed no visible signs of brown spots which signal the immunohistochemical staining of apoptosis (Figure No.2). In the 400X magnification, there is the visible presence of apoptosis, however with a very little amount (Figure No.3).

This result is very different from the histopathologic observation in Group I (where CCl4was administered without giving the soy extract), where at 40X magnification in TUNEL immunohistochemical staining, there are many brown spots in the pherycentral area, indicating the number of cells undergoing apoptosis (Figure No.4).

From the existence of the same histopathologic results between Groups III-VI, with Group II as the negative control of apoptosis, a real difference in appearance is shown in histopathologic observations

of Group I, which was the positive control of apoptosis. From this it can be deduced that the provision soy extract successfully prevented cell apoptosis in the damaged liver tissue of mice (where damage was induced by the administration of CCl4).

Statistical Analysis

The results of the ANOVA test, with 95% confidence level in the results, indicate that the number of apoptotic cells in the group fed soy extract, are less than those in groups not given soy extract, in the liver tissue of mice where injury was induced by CCl4, with $p < 0.001$ (Table No.1). These results demonstrate that the administration of soy extract can prevent the occurrence of apoptotic cells in injured liver tissue in mice.

In this study both the histopathologic observations and statistical analyses have proven that the administration of soy extract at particular level scan prevent liver cell apoptosis in mouse liver tissue where injury is induced by CCl4. This may imply that the administration of soy can provide protection against liver damage, with its ability to prevent cell apoptosis in liver injury circumstances. This is consistent with results of previous studies which have stated that the content of is flavones in soy may reduce oxidative stress levels, and increase the production of nitric oxide (NO) as an anti-apoptosis, by increasing the expression of nitric oxide synthase (NOS) and the production of TNF-α, where by inhibiting the extrinsic pathway initiation there is a limiting of the occurrence of apoptosis[3, 17]. This result is also in accordance with previous research which states that the content of phytoestrogens in soy may also stimulate the production of anti-apoptotic proteins of the Bcl-2 class, so it can also inhibit the initiation of the apoptotic process through the intrinsic pathway (mitochondrial pathway)[17]. The ability of soy to inhibit the initiation of both the extrinsic and intrinsic apoptotic processes in pathways in hepatocytes is what ultimately could play a role in improving survival in conditions in a state of liver injury.

Table No.1: The difference of the amount of apoptotic cell in liver injury induced by CCl4 between the control group and soy treatment groups

S.No	Group	Number of apoptotic cell		
		Mean(SD)	Median	P value*)
1	Group I	76.20(43.01)	92.00(0-104)	**<0,001**
2	Group III	2.20(0,83)	2.00(1.00-3,00)	
3	Group IV	2.20(1.48)	2.00(0.00-4.00)	
4	Group V	2.20(0.84)	2.00(1.00-3.00)	
5	Group VI	2.20(0.71)	2.00(1.00-3.00)	

ANOVA: Analysis of Variants

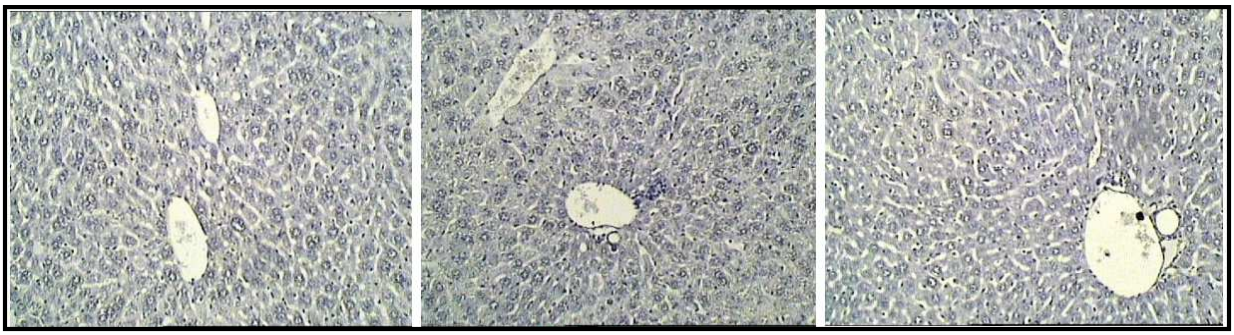
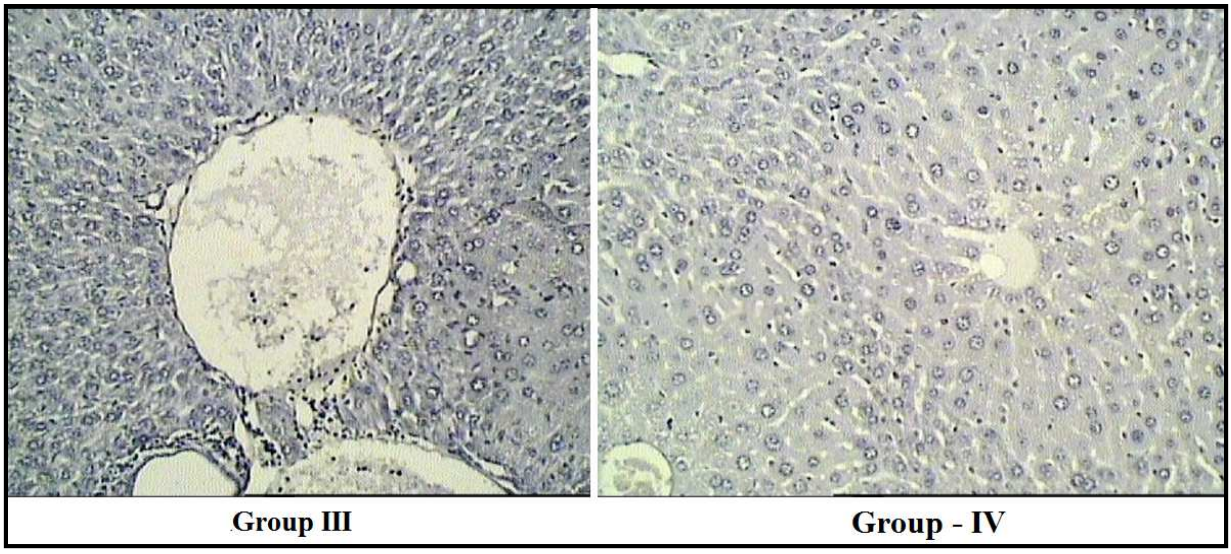


Figure No.1: Results of immunohistochemical TUNEL examination of Group II (negative control apoptosis). No visible signs of apoptosis (brown areas)



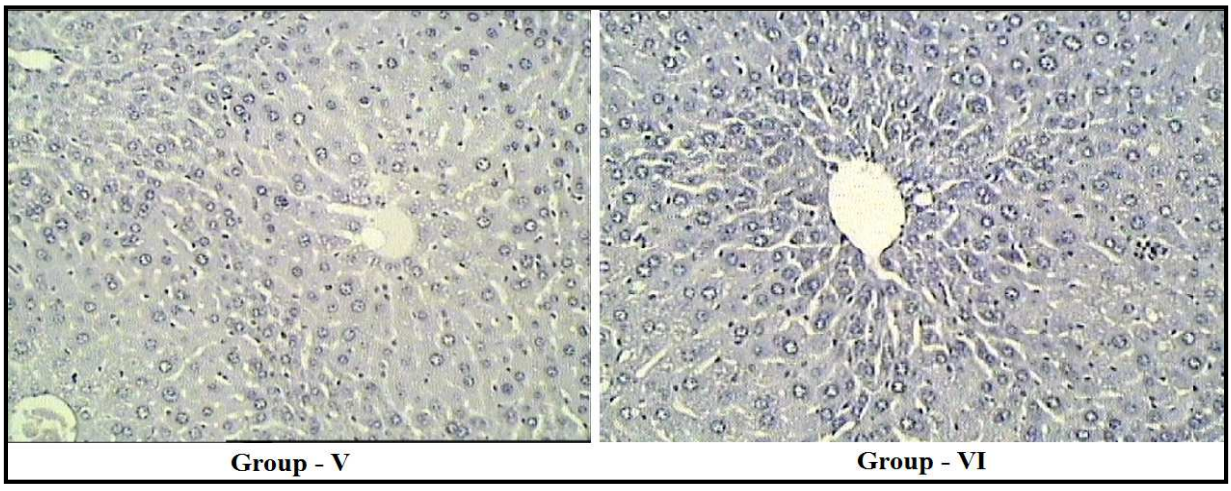


Figure No.2: Results of immunohistochemical TUNEL examination in Groups III-VI, looking the same as Group II who did not show signs of apoptosis (brown areas)

Figure No.3: Immunohistochemical examination of liver tissue by TUNEL in treatment group with 400X magnification. It appears that there is only one cell undergoing apoptosis

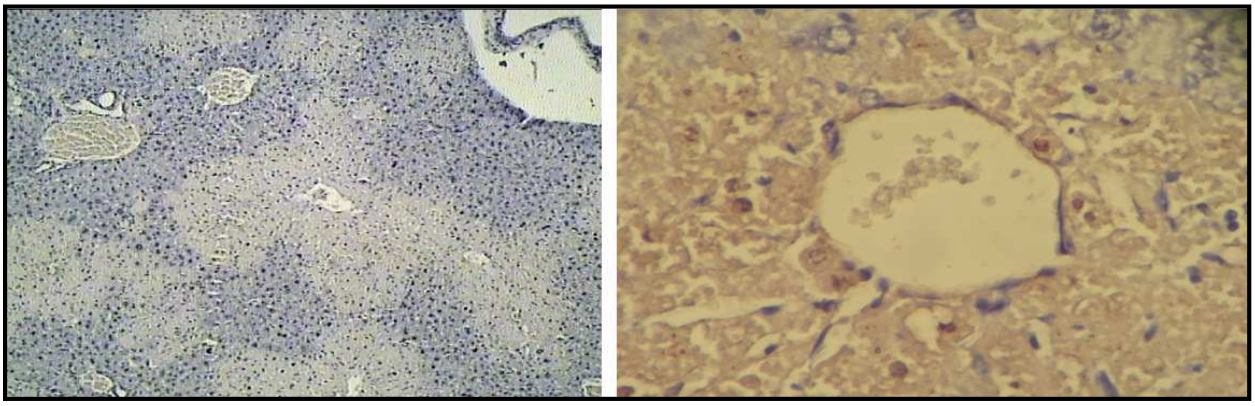


Figure No.4: Results of immunohistochemical TUNEL examination of Group I which is a positive control of apoptosis. There are many brown areas indicating the number of cells undergoing apoptosis

CONCLUSION

From this study it can be concluded that administration of soy within a given range of levels is able to prevent the occurrence of apoptotic cells in injured liver tissue.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

REFERENCES

1. Kumar, Abbas, Fausto. Pathologic Basic of Disease, *Elseviers Saunders, Philadephia,* 7th edition, 2005.
2. Williams G, Iatropoulos M. Alteration in liver function and proliferation: differentiation

between adaptation and toxicity, *Toxicology Pathology*, 30(1), 2002, 41-53.
3. Luedde T, Trautwein C. Intracellular survival pathways in the liver, *Liver International*, 26(10), 2006, 1163-1174.
4. Shi Jialan, Aisaki K, Ikawa Y, Wake K. Evidence of Hepatocyte Apoptosis in Rat Liver after the Administration of Carbon Tetrachloride, *AMJ Pathol*, 153(2), 1998, 515-525.
5. Lim K T, Chae H S, Yoon S H, Kim S S, Kim K Y, Lee W B. Apoptosis in Rat Liver Development: tTG and TGF beta1 Expression Related to Apoptotic Mechanism, *Korean J Anat*, 32(4), 1999, 543-552.
6. Tang X, Gao J, Chen J, Xu L, Tang Y, Dou H, *et al*. Expression of VDAC Regulated by Extracts of *Limonium sinense Ktze* Root against CCl4-Induced Liver Damage, *Int J Mol Sci*, 8(3), 2007, 204-213.
7. Dalton S R, Lee S M L, King R N, Nanji A A, Kharbanda K K, Casey C A, *et al*. Carbon Tetrachloride-Induced liver Damage in Asialoglycoprotein Receptor-Deficient Mice, *Biochemical Pharmacology J*, 77(7), 2009, 1283-1290.
8. Kim S H, Chu H J, Kang D H, Song G A, Cho M, Yang U S. NF-κB Binding Activity and Cyclooxygenase-2 Expression in Persistent CCl4-Treated Rat liver Injury, *JMRI*, 27(3), 2006, 215-223.
9. Etuk E U, Agaie B M, Ladan M J, Garba I. The Modulatory Effect of *Cochlospermum tinctorium* A Rich Aqueous Root Extract on Liver Damage Induced by Carbon Tetrachlorie in Rats, *African Journal of Pharmacy and Pharmacology*, 3(4), 2009, 151-157.
10. Girish C, Koner B C, Rao R, Jayanthi S, Rajesh B, Pradhan S C. Hepatoprotective Activity of Six polyherbal Formulations in CCl4-induced Toxicity in Mice, *Indian journal of Experimental Biology*, 27(4), 2009, 257-263.
11. Nasrin Aghel, Iran Rashidi and Amir Mombeini. Hepatoprotective Activity of *Capparis spinosa* Root Bark against CCl4 Induced Hepatic Damage in Mice, *Iranian journal of Pharmaceutical Research*, 6(4), 2007, 285-290.
12. National Soybean Research Laboratory at the University of Illinois. Research Reports from the Soy/Swine Nutrition Research Program, 1998-1999, 2000.
13. World Initiative for Soy in Human Health. Composition of Soy, 2009. [Cited 2009, 10; 9.35 pm]. From http://www.wishh.org.
14. United Soybean Board. Soy Nutritional Composition. 2009. [Cited 2009 Jul 21.27 pm]. From http://www.soyconnection.com.
15. Suh K S, Koh G, Park C Y, Woo J T, Kim S W, Kim J W, *et al*. Soybean Isoflavones Inhibit Tumor Necrosis Factor-α-Induced Apoptosis and The Production of Interleukin-6 and Prostaglandin E_2 in Osteoblastic Cells, *J Phytochemistry*, 63(2), 2003, 209-215.
16. Oka I B W, I Nyoman S. Perubahan Morfologi Hati dan Ginjal Mencit Yang Diinduksi Karbontetraklorida (CCl_4), Jurnal Veteriner Fakultas Kedokteran Udayana, 6(1), 2005, 26-31.
17. Blake A, Jones, Gregory, Gores. Physiology and Pathophysiology of Apoptosis in Epithelial Cells of the Liver, Pancreas, and Intestine, *Am J Physiol Gastrointest Liver Physiol*, 273(6), 1997, G1174-G1188.

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