International Journal of Applied Research 2016; 2(5): 558-562

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(5): 558-562
www.allresearchjournal.com
Received: 04-03-2016
Accepted: 05-04-2016

Kshiteej Sode
MSc student Dept. of Life Science and Biochemistry St. Xavier's College 5, Mahapalika Marg Mumbai 400001, India.

Payal Gupta
MSc student Dept. of Life Science and Biochemistry St. Xavier's College 5, Mahapalika Marg Mumbai 400001, India.

Radiya Pacha-Gupta
Associate Professor Dept. of Life Science and Biochemistry St. Xavier's College 5, Mahapalika Marg Mumbai 400001, India.

Seema Das
Associate Professor Dept. of Life Science and Biochemistry, St. Xavier's College 5, Mahapalika Marg Mumbai 400001, India.

Correspondence
Kshiteej Sode
MSc studentDept. of Life Science and Biochemistry St. Xavier's College 5, Mahapalika Marg Mumbai 400001, India.

Cadmium-induced developmental defects in chick embryos and antioxidant effects of green tea

Kshiteej Sode, Payal Gupta, Radiya Pacha-Gupta, Seema Das

Abstract
The heavy metal, cadmium (Cd) is known to be teratogenic leading to malformations in a number of vertebrate systems (Järup and Åkesson, 2009; Adams *et al*., 2011; Nordberg *et al*., 2009; Jin *et al*., 2016) [1-4]. This effect has been attributed to the oxidative stress induced by cadmium (Cuypers *et al*., 2010) [6]. The rationale behind this study was to evaluate Cd induced damage on the morphological and biochemical development of chick embryos and a subsequent rescue of the same using crude green tea extract. Hence, varying concentrations of cadmium chloride were injected in chick embryos on day zero post fertilization (dpf) and then observed for mortality, morphological abnormalities and marker enzymes of oxidative stress. The lowest observed effect level (LOEL) of Cd was found to be 25mM. Blood clots, hemorrhage, microphthalmia, exencephaly, micromelia and arrested development were observed in Cd treated embryos. Activity of oxidative stress marker Alanine aminotransferase (ALT) showed a significant decrease in Cd treated embryos, indicating hepatotoxicity. A significant decrease in total protein concentrations was also observed. Protein profiles showed additional protein bands in treated embryos, indicating cadmium induced changes in protein expression. To investigate possible green tea reversal of toxic effects of cadmium, the embryos were treated with Cd and green tea extracts simultaneously. Rigid, brown masses, not seen in any of the controls, were observed. These brown masses showed a significant increase in total protein concentrations and ALT levels. Embryos treated with only green tea extract, however, showed normal embryonic development and normal levels of proteins and ALT. Thus, the results indicate that green tea was unable to rescue the embryo from the toxic effects of cadmium. We propose that cadmium and green tea play a synergistic role in activating antioxidants which proves detrimental for normal growth and development.

Keywords: Cadmium Toxicity, Chick Embryos, Green tea, Oxidative stress, Antioxidant, ALT

1. Introduction
Cadmium is a heavy metal pollutant made available for consumption through direct sources such as tobacco consumption, or through indirect sources such as food cultivated in groundwater laced with Cadmium (Järup and Åkesson, 2009; Adams *et al*., 2011) [1, 2]. In adults, Cd toxicity can lead to kidney dysfunction as well as bone diseases such as the itai-itai disease (Nordberg *et al*., 2009) [3]. There have also been studies showing an increased risk of congenital heart defects in offspring in Cd exposed pregnancies (Jin *et al*., 2016) [4], as well as a decreased birth rate and increased fetal and maternal glucocorticoids (Ronco *et al*., 2009) [5]. However, the underlying mechanisms of Cd-mediated teratogenicity are not known clearly. Oxidative stress and Cd-induced reactive oxygen species generation is one of the explanations that has come to light after numerous studies (Cuypers *et al*., 2010) [6]. In Jurkat T cell lines, Cadmium exposure has shown to deplete enzymes glutathione and the total intracellular sulfhydryl groups while increasing lipid peroxidation and protein carbonyl levels, indicating oxidative stress (Nemmiche *et al.,* 2011) [7]. Furthermore, hepatic necrosis as well as neurotoxicity caused by Cd-induced oxidative stress has been shown in mice, by altered levels of intracellular trace elements, cofactors of metalloenzymes such as Zinc, and decreased levels of antioxidants such as glutathione (Sinha *et al*., 2009) [8]. There have also been various rescue attempts that have beencarried out against Cd-induced oxidative stress.

Taurine and Vitamin C have shown significant reduction in hepatotoxicity and neurotoxicity in mice (Sinha *et al*., 2008) [9]. Green tea is a known antioxidant, with beneficial effects in a myriad of ailments from liver disease to cancer (Chacko *et al*., 2010) [10]. Hence, the aim of this study is to (1) assess the morphological defects, biochemical changes and oxidative stress levels in early developing chick embryos exposed to cadmium (2) attempt an antioxidant rescue for cadmium toxicity using green tea extracts.

2. Materials and Method

2.1 Procurement of Eggs

Fertilized white leghorn chicken eggs (*Gallus Gallus domesticus*) were purchased from the Central Poultry Breeding Farm, Aarey Milk Colony, Goregaon (East), Mumbai.

2.2 Assignment of Eggs into Study Groups

All eggs were fertilized on the day of purchase (0 days post fertilization). These eggs were cleaned and segregated into different study groups.

2.3 Treatment of Embryos with Cadmium Chloride and Green tea

The dosage for $CdCl_2$was set within a concentration range of 10mM to 125mM. $CdCl_2$ was dissolved in Phosphate Buffer Saline (PBS) to obtain concentrations of 10, 25, 50, 75,100 and 125mM. Each study group had a negative control which was not injected and a technical control which was injected with avian saline (0.75% NaCl). Subsequently, the Hamburger-Hamilton (HH) stages of chick embryo development were used to stage the chick embryos. Cadmium administration was carried out on day 0 post fertilization. Treatments were made through a hole created in the air sac of every egg using a sterile needle.

The first three batches of eggs were given 20 µL of $CdCl_2$ into each treatment egg, and the same volume of avian saline was injected into the technical control egg of every batch. The fourth batch was given 100 µL of Lipton Green tea crude filtrate in dilutions of 1:10, 1:100 and 1:1000 half an hour after administration of $CdCl_2$. All eggs were incubated at 37 °C.

2.4 Morphological Analysis

All embryos were dissected for morphological examination 2, 4, 5, 6, 7, 8, 9 and 10 days post-fertilization (HH stages 21-36). The embryo was extracted by careful removal of albumin from the egg without disturbing the embryo, followed by removal of the embryo itself using blunt forceps and mounting onto a petri-dish. The embryo was bathed in PBS in order to prevent drying up. Each embryo was examined under a camera mounted dissection microscope for deformities. The heartbeat count of the chick embryos was also noted at the time of morphological analysis. After examination, the inner mass was extracted from embryo for protein analysis.

2.5 Protein Extraction

The liver was weighed and subsequently homogenized on ice in Laemmli's protein extraction buffer (100 ml composition of 2X solution: 17.5 ml of 20% w/v SDS, 5ml beta-mercaptoethanol, 10ml Glycerol, 6.3ml 1M Tris-Cl at pH 6.8, 61.2ml distilled water). The homogenate was immediately transferred to a heat block and kept at 80 °C for 1 minute. The heated samples were transferred to ice till they cooled down, and subsequently centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was separated out and stored at -20 °C for enzyme assays and SDS-PAGE.

2.6 Enzyme Assays

ALT and total protein was analyzed using ERBA-Manheim Enzyme Assay Kits. All tests were conducted on the liver extract 24 hours post embryo dissection, during which time the liver was stored at -20 °C.

2.7 Protein Profiles

The extract was used for SDS-PAGE analysis to study any Cd induced differences in protein expression.

3. Results

Table 1: Comprehensive Evaluation of the Morphological Effects of Cd and attempted Green Tea Rescue on Chick Embryos

	Control	Cadmium Treated	Green Tea	Cadmium+ Green Tea
Live Embryos	94%	72.36%	100.00%	0.00%
Dead Embryos	6.45%	27.64%	0.00%	100%
Heart Rate (Median)	83 ± 11	39.62 ± 21.53	84 ± 9	Not observed
Abnormal embryos	NA	31.71%	NA	100%
Blood Clots/ Hemorrhage	NA	33.33%	NA	0.00%
microphthalmia	NA	64.10%	NA	13.00%
Exencephaly	NA	5.13%	NA	0.00%
Micromelia	NA	28.21%	NA	0.00%
Arrested Development	NA	43.59%	NA	9.73%
Brown Masses	NA	Not observed	NA	77.27%

NA: no abnormalities observed

3.1 Morphological Analysis

In Cd treated eggs, a number of morphological abnormalities were observed. Largely, a decrease in the total number of heart beats per minute was observed in Cd treated embryos as compared to the control groups (Table 1). 25mM was established as the non-lethal LOEL amongst all groups. The most commonly observed defects included blood clots, hemorrhage, microphthalmia, exencephaly, micromelia and arrested development (Table 1). These abnormalities in Cd treated embryos were seen five days post fertilization (HH stage 26) onwards (Fig 1: M1-M7). Green Tea treated embryos showed no observable abnormalities in the morphology, whereas Cd and Green Tea treated embryos showed formation of dead brown masses instead of distinct living embryos (Fig 1: M15)

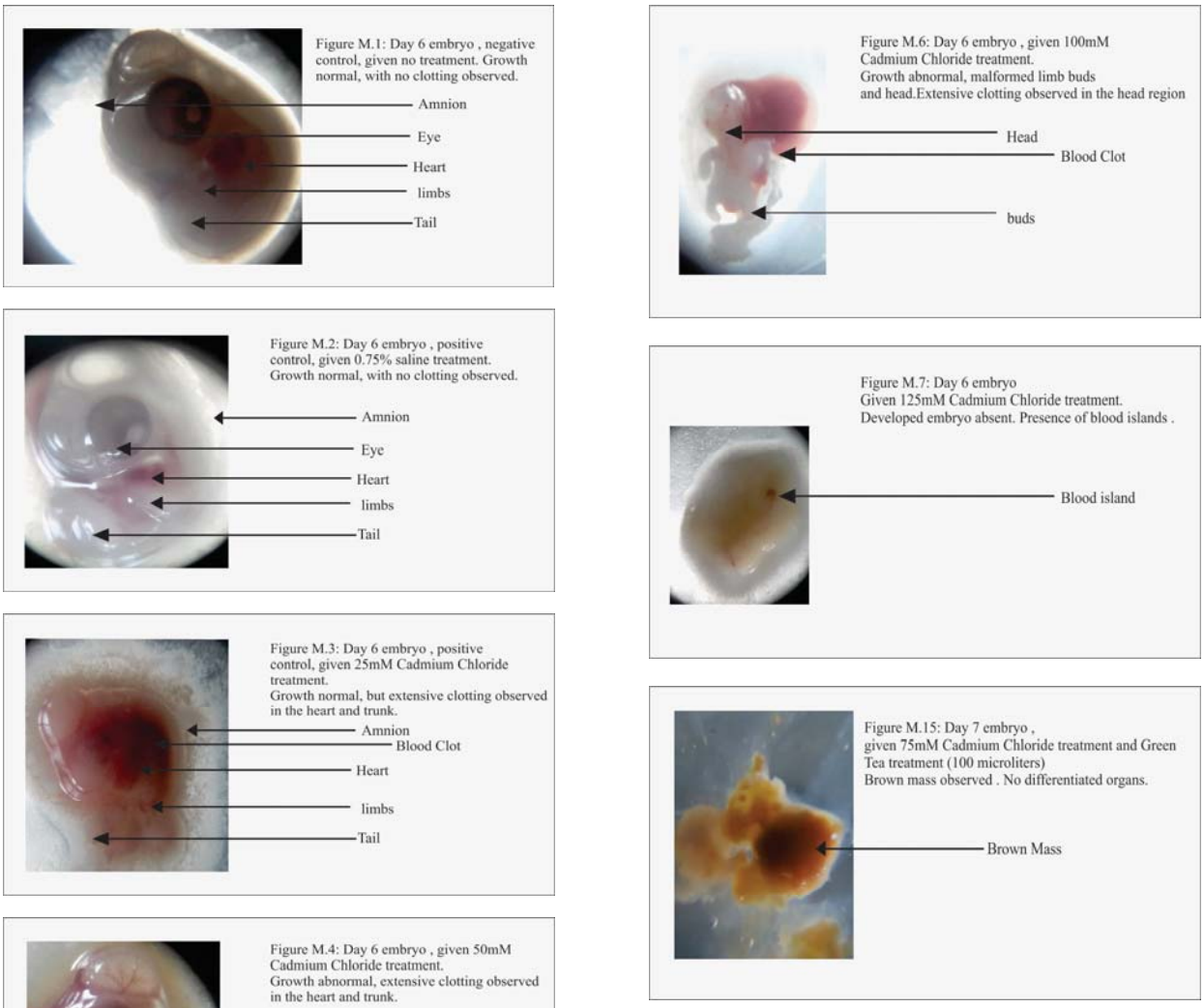

Fig 1: Morphological observations of chick embryos treated with $CdCl_2$ (25mM-125mM) in comparison with the negative and technical controls.

3.2 ALT and Total Protein Assays

ALT levels showed a decrease with increasing cadmium concentrations as compared to the control (Fig 2). A paired t-test was conducted to compare ALT Levels in Cd treated embryos and control embryos (Table 2). The test showed significant difference between the Cd tested groups and the control groups. With increasing concentration, the t-score increased and the p-level decreased, showing an overall increase in the significance of the data. Total protein levels showed a similar trend with increasing concentrations of $CdCl_2$. ALT levels and total protein levels showed a significant increase in Cd and green tea as compared to Cd treated counterparts (Table 3).

Table 2: Mean Paired t- test of ALT and Total protein concentrations between Cd treated samples and negative control samples

	Concentration of $CdCl_2$	dF	Negative Control Samples (IU)	Cd treated samples(IU)	t-score	p-level*
ALT	25mM	55	1012.18 ± 651.11	308.71 ± 89.19	3.28	0.18%
	50mM	52	1036.60 ± 640.10	290.03 ± 89.41	3.83	0.02%
	75mM	55	1052.30 ± 640.62	128.0 ± 44.16	5.21	0.00%
	Concentration of $CdCl_2$	**dF**	**Negative Control Samples (g/dL)**	**Cd treated samples(g/dL)**	**t-score**	**p-level***
Total Protein	25mM	55	15.71 ± 5.29	7.61 ± 4.19	4.01	0.02%
	50mM	52	16.40 ± 4.94	7.39 ± 3.74	4.8	0.00%
	75mM	55	15.79 ± 5.19	4.61 ± 2.79	5.15	0.00%

*p-levels below 5% indicate statistically significant results

Table 3: Paired t- test of ALT and Total protein concentrations between Cd treated samples, and Cd and Green tea treated samples.

	Concentration of $CdCl_2$	dF	Cd and Green tea (IU)	Cd treated samples(IU)	t-score	p-level*
ALT	25mM	23	489.67 ± 104.2	308.71 ± 89.19	5.37	0.00%
	50mM	20	385.97 ±68.23	290.03 ± 89.41	2.99	0.72%
	75mM	20	916.08 ± 245.09	148.65 ± 65.79	2.88	0.93%
	Concentration of $CdCl_2$	**dF**	**Cd and Green tea (g/dL)**	**Cd treated samples(g/dL)**	**t-score**	**p-level***
Total Protein	25mM	23	10.59 ± 1.27	6.78 ± 4.01	4.29	0.03%
	50mM	20	10.76± 1.14	6.24 ± 3.46	3.18	0.47%
	75mM	20	12.53 ± 1.07	3.96 ± 2.09	4.14	0.05%

*p-levels below 5% indicate statistically significant results

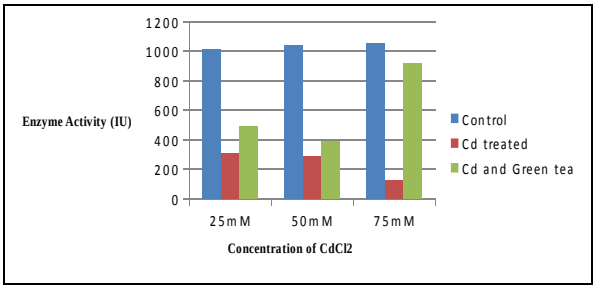

Fig 2: ALT activity in chick embryos subjected to various treatments. Negative control results are shown.

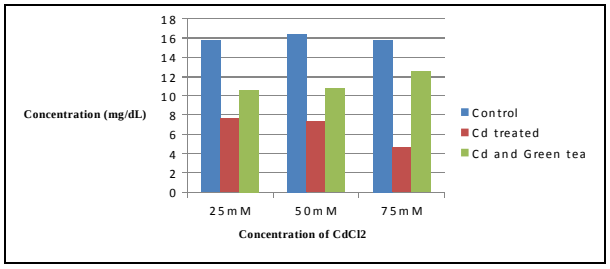

Fig 3: Total protein concentrations in chick embryos subjected to various treatments. Negative control results are shown.

3.3 SDS-PAGE Analysis

SDS-PAGE Analysis revealed three new protein bands in the Cd treated samples. These protein bands were not seen in control samples. A third extra protein band was seen in Cd and green tea treated samples. In Cd treated samples, two of the protein bands showed increasing concentrations (Fig. 4 and Fig. 5).

Lane Number	Sample
1	Saline treated technical control 5 dpf
2	Negative control 5 dpf
3	1:10 Green tea + 25mM Cd treated 5 dpf
4	1:10 Green tea + 50mM Cd treated 5 dpf
5	1:10 Green tea + 75mM Cd treated 5 dpf
6	Saline treated technical control 5 dpf
7	Negative control 5 dpf
8	25mM Cd treated 5 dpf
9	50mM Cd treated 5 dpf
10	75mM Cd treated 5 dpf

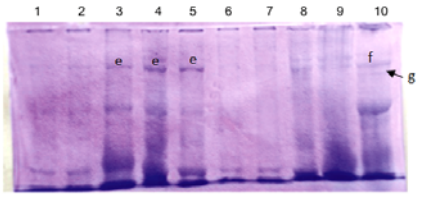

Fig 4: SDS-PAGE showing Cd and green tea treated samples in lanes 1-5, in comparison with samples treated with Cd in lanes 6-10, along with their respective technical (saline treated) and negative (untreated) counterparts. The band marked 'e' is absent in the control samples, and the intensity of the bands increase as the concentration of cadmium increases. The presence of bands 'g' and 'f' are marked in Cd treated samples. These bands are absent in control samples. The 'e' band in green tea and cadmium treated samples is almost in the same position as the 'f' band, but the 'g' band is not observed in lanes 3, 4, and 5.

Lane Number	Sample
1	Negative control 7 dpf
2	Saline treated technical control 7 dpf
3	50mM Cd treated 7 dpf
4	75mM Cd treated 6 dpf
5	75mM Cd treated 7 dpf sample
6	1:10 Green tea + 25mM Cd treated 7 dpf
7	1:10 Green tea + 50mM Cd treated 7 dpf
8	1:10 Green tea + 50mM Cd treated 7 dpf
9	1:10 Green tea + 50mM Cd treated 6 dpf

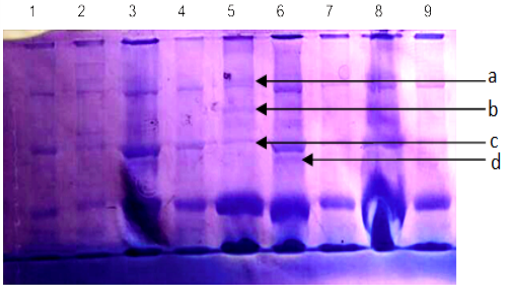

Fig 5: The figure shows a comparison between the control (saline treated) and Cd treated samples in comparison with Cd and Green tea treated samples. The most prominent difference was the appearance of two additional protein bands in the Cd as well as Cd and green tea treated samples. These bands have been labeled bands 'c' and'd' respectively. Samples treated with Green tea showed two additional bands as compared to untreated and control samples which have been labeled as 'a' and 'b'.

4. Discussion

While the morphological findings showed a number of different abnormalities, these were consistent with previous studies on Cd treated chick embryos (Veeriah *et al*., 2014) [11]. However, there has been no report on any brown masses observed with the addition of antioxidants in green tea. The significance of this study needs to be explored further. Cadmium has also been shown to induce oxidative stress in vertebrate embryos (Sinha *et al*., 2009) [8]. Our study showed a significant decrease in oxidative stress marker enzyme ALT. This could also be an indicator of hepatotoxicity (Ghouri *et al*., 2010; Wang *et al*., 2012) [12, 13]. Changes in protein profiles observed in treated vs. untreated embryos indicate possible changes in gene expression. Microarray analysis could be used to look for changes in gene expression.

5. Conclusion

Developmental abnormalities were observed in Cd treated chick embryos. Biochemical analysis showed a significant decrease in total protein and ALT levels. Chick embryos treated with green tea did not show any abnormalities or mortalities, and were morphologically and biochemically normal. Living embryos were not observed in the presence of green tea in Cd treated embryos.

6. Acknowledgements

This work was funded by the Department of Life Science and Biochemistry, St. Xavier's College, Mumbai.

7. References

1. Järup L, Åkesson A. Current status of cadmium as an environmental health problem. Toxicology and Applied Pharmacology 2009; 238(3):201-208. http://dx.doi.org/10.1016/j.taap.2009.04.020
2. Adams S, Newcomb P, Shafer M, Atkinson C, Bowles E, Newton K *et al*. Sources of cadmium exposure among healthy premenopausal women. Science of the Total Environment 2011; 409(9):1632-1637. http://dx.doi.org/10.1016/j.scitotenv.2011.01.037
3. Nordberg G. Historical perspectives on cadmium toxicology. Toxicology and Applied Pharmacology 2009; 238(3):192-200. http://dx.doi.org/10.1016/j.taap.2009.03.015
4. Jin X, Tian X, Liu Z, Hu H, Li X, Deng Y *et al*. Maternal exposure to arsenic and cadmium and the risk of congenital heart defects in offspring. Reproductive Toxicology 2016; 59:109-116. http://dx.doi.org/10.1016/j.reprotox.2015.12.007
5. Ronco A, Urrutia M, Montenegro M, Llanos M. Cadmium exposure during pregnancy reduces birth weight and increases maternal and foetal glucocorticoids. Toxicology Letters 2009; 188(3):186-191. http://dx.doi.org/10.1016/j.toxlet.2009.04.008
6. Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H *et al*. Cadmium stress: an oxidative challenge. Biometals 2010; 23(5):927-940. http://dx.doi.org/10.1007/s10534-010-9329-x
7. Nemmiche S, Chabane-Sari D, Kadri M, Guiraud P. Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. Toxicology *in vitro* 2011; 25(1):191-198. http://dx.doi.org/10.1016/j.tiv.2010.10.018
8. Sinha M, Manna P, Sil P. Induction of necrosis in cadmium-induced hepatic oxidative stress and its prevention by the prophylactic properties of taurine. Journal of Trace Elements in Medicine and Biology. 2009; 23(4):300-313. http://dx.doi.org/10.1016/j.jtemb.2009.03.010
9. Sinha M, Manna P, Sil P. Cadmium-induced neurological disorders: prophylactic role of taurine. J Appl. Toxicol, 2008. http://dx.doi.org/10.1002/jat.1363
10. Chacko S, Thambi P, Kuttan R, Nishigaki I. Beneficial effects of green tea: A literature review. Chinese Medicine 2010; 5(1):13. http://dx.doi.org/10.1186/1749-8546-5-13
11. Veeriah V, Saran U, Swaminathan A, Balaguru U, Thangaraj P, Nagarajan S *et al*. Cadmium-Induced Embryopathy: Nitric Oxide Rescues Teratogenic effects of cadmium. Toxicological Sciences 2014; 144(1):90-104. http://dx.doi.org/10.1093/toxsci/kfu258
12. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: A narrative review and clinical perspective of prospective data. Hepatology 2010; 52(3):1156-1161. http://dx.doi.org/10.1002/hep.23789
13. Wang C, Chang T, Yao W, Wang S, Chou P. Impact of increasing alanine aminotransferase levels within normal range on incident diabetes. Journal of the Formosan Medical Association. 2012; 111(4):201-208. http://dx.doi.org/10.1016/j.jfma.2011.04.004