



## The Effects of *in ovo* Nanocurcumin Administration on Oxidative Stress and Histology of Embryonic Chicken Heart

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### Abstract

This study was designed to evaluate the effects of nanocurcumin (NC) on oxidative stress and histology of embryonic chicken heart. NC was injected into the yolk of 4-day-old embryonic eggs at one of three doses: 10 ppm (NC10 group), 100 ppm (NC100 group), and 1000 ppm (NC1000 group). The control group received normal saline. Oxidative stress in heart tissue was evaluated by measuring malondialdehyde (MDA) concentration, glutathione (GSH) content, and ferric reducing antioxidant power (FRAP). Serum lipids and cardio-histopathology were also measured. There were no significant differences in GSH, FRAP, and MDA levels between the control and treatment groups ( $P > 0.05$ ). The serum lipid profile was altered in the NC100 group, with reduced levels of triglyceride (TG) ( $P < 0.01$ ) but higher levels of HDL-c ( $P < 0.01$ ) compared to the control. Heart histology was similar between NC10 and NC100 treatments compared to the control group. However, heart sections in NC1000 revealed focal areas of disrupted cardiac muscles and mild infiltration of mononuclear inflammatory cells between muscle fibers. It was concluded that NC at a concentration of 100 ppm did not damage heart tissues in chicken embryo and could be used as a valuable molecule for cardiovascular disease prevention.

### Introduction

Curcumin is a yellow pigment and hydrophobic polyphenol derived from *Curcuma longa*, a plant used as a spice, food coloring, medical preparation, and cosmetic compound (Anand *et al.*, 2007; Menon and Sudheer, 2007; Wu *et al.*, 2007). Human (Satoskar *et al.*, 1986; Ramsewak *et al.*, 2000; Menon and Sudheer, 2007) and animal studies (Nabavi *et al.*, 2011; Nautiyal *et al.*, 2011; Yallapu *et al.*, 2012) have detected anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-infection, hypocholesterolaemic and cardio-protective properties of curcumin. Curcumin

could be also a therapeutic choice for the treatment of diabetes and neurodegenerative disease (Ghosh *et al.*, 2015).

Animal models and human studies have confirmed that dietary curcumin is safe and does not have side effects, even at high doses (Lao *et al.*, 2006; Ganiger *et al.*, 2007; Wu *et al.*, 2007; Chen *et al.*, 2010). However, few experiments have studied the potential effects of curcumin on embryo development. Some work has demonstrated embryo-toxic and teratogenic effects of curcumin on embryonic development

in mouse and zebrafish models (Wu *et al.*, 2007; Huang *et al.*, 2013). In an *in vitro* study, JiangHua *et al.* (2013) revealed that buffalo zygotes displayed developmental defects when exposed to high-dose curcumin (20  $\mu$ M).

Regardless of the positive effects of curcumin, some studies have shown that curcumin is insoluble in water and has low bioavailability (Yang *et al.*, 2007; Shaikh *et al.*, 2009; Yallapu *et al.*, 2012). These properties of curcumin are limiting factors that can cause low absorption, fast metabolism, and fast systemic elimination from the body. Hence, many types of curcumin have been produced including nano formulations (Liu and Chang, 2011; Liu *et al.*, 2013). Some studies in cell culture, animal models, and healthy subjects showed that nanocurcumin (NC) is neutral and has no detrimental effects in high concentrations (Pourasgari *et al.*, 2009; Kanai *et al.*, 2012; Sarbolouki *et al.*, 2012). A literature review also showed that NC has therapeutic efficacy as an anti-inflammatory, anticancer, immunomodulatory, and neuroprotective agent (Bisht *et al.*, 2007; Wang *et al.*, 2008; Kakkar and Kaur, 2011; Jain *et al.*, 2013; Kakkar *et al.*, 2013; Sankar *et al.*, 2013).

Nonetheless, the consequences of NC administration to embryos remain unclear. Considering the higher efficiency of NC than curcumin, it is very important to evaluate any possible toxicological risk of this product. In this study, we used chicken embryo as an experimental model to investigate the developmental cardio toxicity of NC.

### Materials and Methods

Nanocurcumin was obtained from ExirNanoSina in Tehran, Iran (IRC: 1228225765). This compound (SinaCurcumin<sup>®</sup>) has been produced in Nanotechnology Research Center of Mashhad University of Medical Sciences, Mashhad, Iran.

### Experimental design

The experiment was conducted in accordance to protocols approved by Animal Care Committee of Amol University of Special Modern Technologies, Mazandaran, Iran. 60 fertile eggs were obtained from a traditional breeding farm and divided into four groups. The control group received normal saline, and three experimental groups received nanocurcumin in one of three doses: 10 ppm (NC10), 100 ppm (NC100), or 1000 ppm NC (NC1000). The selected doses

were chosen based on our previous pilot study (data not published). On the fourth day of incubation, the treatment groups received 0.1 ml of the appropriate dose of NC into the yolk sac while the control group received 0.1 mL saline solution according to methods described by McLaughlin *et al.* (1963). The injection sites were covered by paraffin and the eggs were incubated at 37-38°C and 60% relative humidity in a forced draught incubator (Noiva *et al.*, 2014).

The eggs were candled one day after injection and then checked every 48 hrs. All 60 fertile eggs hatched on the 21<sup>st</sup> day of incubation. Blood samples were collected by direct cardiac puncture and sera were obtained by centrifugation at 2800  $\times$  g for 15 min. Half of the heart tissue from the newly hatched chicks was removed (n = 60) and fixed in 10% buffered formalin for histopathological examination, and the other half was stored at -20°C until used to assess oxidative stress (n = 60) (Seifi *et al.*, 2015). Heart tissues were homogenized in 10x (w/v) sodium phosphate buffer. The homogenate was centrifuged at 1008  $\times$  g for 15 min, and the supernatant was used to measure indices of oxidative stress.

### Measurement of lipid peroxidation

Lipid peroxidation in chick serum was determined by measuring malondialdehyde (MDA) using a thiobarbituric acid reactive substances (TBARS) assay (Abe *et al.*, 2014). Briefly, the supernatant of the egg yolk was mixed with 20% trichloroacetic acid, and then centrifuged at 2800  $\times$  g for 5 min. Then, thiobarbituric acid was added to the supernatant and incubated for 90 min in a 90°C water bath and cooled down to room temperature. The absorbance was measured at 532 nm. The values are expressed in nmol MDA, using a molar extinction coefficient of  $1.56 \times 10^5$  MG<sup>-1</sup> cmG<sup>-1</sup> (Sadighara *et al.*, 2013).

### Measurement of total glutathione (GSH) content

Glutathione content in heart tissues was measured according to methods described by Gibson *et al.* (1998). The samples were rinsed three times with phosphate buffered saline and mixed with 20% trichloroacetic acid. After centrifugation at 2800  $\times$  g for 5 min, the supernatant was mixed with 4 volumes of Tris buffer. 1 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] was then added to the

samples and incubated for 30 min. Absorbance was read at 412 nm.

#### Ferric reducing antioxidant power (FRAP) measurement

The antioxidant capacity of heart tissue samples was determined by measuring the ability of samples to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . The combination between  $\text{Fe}^{2+}$  and 2, 4, 6-tris-(2-pyridyl)-1, 3, 5-triazine (TPTZ) gave a blue color and absorbance was read at 593 nm (Benzie et al., 1996).

#### Serum lipid profile determination

Total cholesterol content was estimated according to methods described by Turley et al. (1994). Low density lipoprotein cholesterol (LDL-c) and high density lipoprotein cholesterol (HDL-c) were measured in serum according to the Wyne and Woollett (1998) method. Triglyceride (TG) content was measured using modified methods from Hermann et al. (2000).

#### Statistical analysis

All experimental samples were repeated in triplicate. All results are expressed as mean  $\pm$

SD. Statistical analyses were performed by employing Student's t test for unpaired data using the SPSS (2006) 16.0 software package. Significance was established at the  $P < 0.05$  level.

#### Results and Discussion

There were no gross abnormalities in chicken embryos after necropsy. Oxidative stress variables including MDA, FRAP, and GSH are shown in Table 1. Levels of MDA and antioxidants were similar between NC treatment groups and the control group ( $P > 0.05$ ). Although GSH content was higher in the groups exposed to NC than the control, this difference was insignificant ( $P > 0.05$ ). Glutathione is a tripeptide antioxidant that protects tissues against oxidative damage (Kidd, 1997). Flora et al. (2013) reported no changes in GSH content and TBARS (a lipid peroxidation marker) levels in mice treated with nanocurcumin (15 mg/kg, orally) throughout the experimental period of 14 days. Another study demonstrated that nanocurcumin-treated cardiomyoblasts H9c2 cells had reduced lipid peroxidation without alterations in GSH (Nehra et al., 2015).

**Table 1.** Effects of nanocurcumin on antioxidant status in heart of chicken embryo

Groups	MDA	GSH	FRAP
	(nmol/g sample)	( $\mu\text{mol/g}$ sample)	(mmol/g sample)
Control	0.17 $\pm$ 0.05	0.28 $\pm$ 0.12	1.044 $\pm$ 0.46
NC10	0.18 $\pm$ 0.05	0.036 $\pm$ 0.13	0.903 $\pm$ 0.25
NC100	0.18 $\pm$ 0.03	0.31 $\pm$ 0.10	1.32 $\pm$ 0.38
NC1000	0.19 $\pm$ 0.01	0.33 $\pm$ 0.11	1.27 $\pm$ 0.34

Values are presented as Mean  $\pm$  SD

Control group: without injection of Nanocurcumin, NC10, NC100 and NC1000 groups were injected with 10, 100, and 1000 ppm NC into the yolk.

The effects of nanocurcumin on the serum lipid profile are shown in Table 2. The levels of TC and LDL-c were similar across all groups ( $P > 0.05$ ). Although TG levels were lower in the NC treated groups than the control group, this difference was only significant in the NC100 group ( $P < 0.01$ ). The levels of HDL-c were significantly ( $P < 0.01$ ) higher in NC100 group compared to the control and NC10 groups. In line with the present study, Rahimi et al., (2016) showed an increase in HDL-c and decrease in TG in diabetic patients that received 80 mg/day of curcumin as nano-micelle. Several studies have shown that curcumin may have a protective effect against cardiovascular disease like myocardial infarction, hypertension, and diabetic cardiomyopathy,

and NC has been proven to be effective in treatment of different diseases such as cardiovascular disease (Nabavii et al., 2011; Yallapu et al., 2012; Nehra et al., 2015; Xiao et al., 2016). Moreover, protective effects of curcumin on serum lipid fractions were also shown in healthy humans (Soni and Kuttan, 1992), experimental hepatic fibrosis (Akila et al., 1998), and normal and hyper lipidemic rats (Ghada, 2005).

Histopathological evaluations revealed that the heart sections from NC10 and NC100 groups were similar to the control group and displayed normal appearance (Fig. A, B, C). In brief, cardiac muscle fibers were well arranged with centrally located nuclei. Connective tissue also appeared normal. Examination of the heart sections in the NC1000 group revealed focal

areas of disrupted cardiac muscle fibers, mild degeneration changes, and fragmentation in the cardiac muscle fibers (Figure D). Mild infiltration of mononuclear inflammatory cells was also detected between muscle fibers. Similar

to our results, Ding et al. (2014) found intravenous administration of Curcumin Poly (ester amine) nanoparticles at low concentration (25 ppm) in the BALB/c mice did not induce any histological damage in heart tissue.

**Table 2.** Effects of nanocurcumin levels on serum lipid profile of chicken embryo

Groups	TC	TG	HDL-c	LDL-c
	mg/dL	mg/dL	mg/dL	mg/dL
Control	448.45 ± 78.39	105.48 ± 33.05	134.25 ± 20.31	336.75 ± 119.54
NC10	483.27 ± 80	64.2 ± 23.32	127.25 ± 20.02	406.81 ± 149.87
NC100	399.91 ± 68	42.63 ± 12.37*	173.88 ± 19.77**	252.77 ± 109.14
NC1000	477.34 ± 71.50	52.8 ± 18.45	148.00 ± 7.87	344.75 ± 128.42

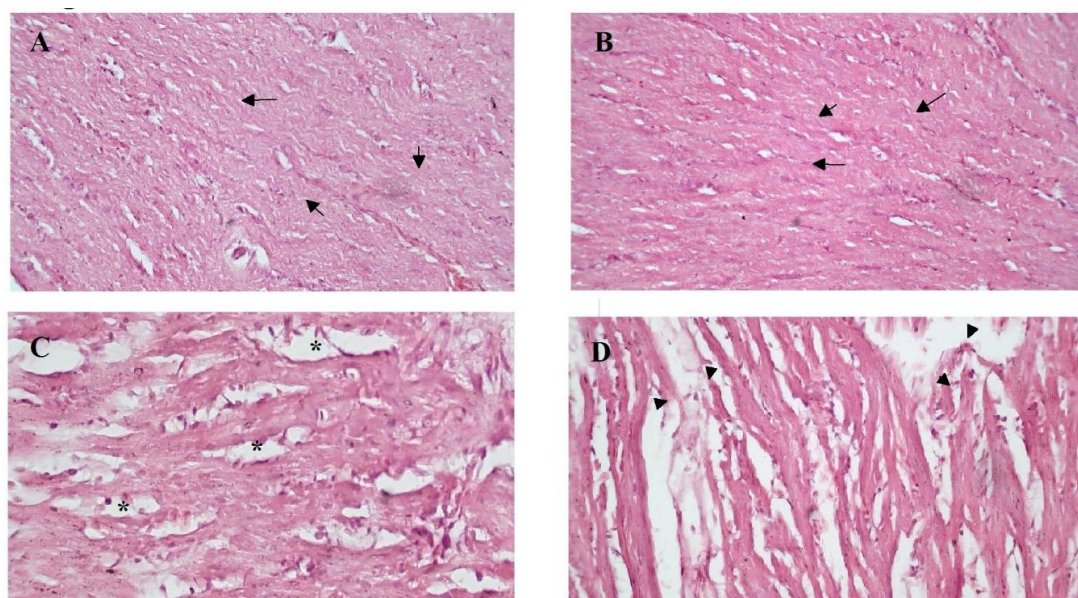
Values are presented as Mean ± SD.

Control group: without injection of Nanocurcumin, NC10, NC100 and NC1000 groups were injected with 10, 100, and 1000 ppm NC into the yolk.

\*Significantly decreased in NC100 group compared with that in control group ( $P < 0.01$ ).

\*\*Significantly increased in NC 100 group compared with that in NC 10 and control groups ( $P < 0.01$ ).

Total cholesterol (TC), Triglyceride (TG), Low density lipoprotein cholesterol (LDL-c) and High density lipoprotein cholesterol (HDL-c).



**Figure 1.** Histopathological evaluation of the heart sections. **(A):** Control group, well-arranged cardiac muscle fibers (arrow) (H & E, ×100). **(B):** NC 10 group, normal cardiac structure with the same appearance compared to control group. Well-organized muscle fibers can be detected with arrows (H & E, ×100). **(C):** NC 100 group, normal cardiac structure and many vascular structure (star) exist between cardiac muscles (H & E, ×400). **(D):** NC1000 group, focal areas of disrupted cardiac muscle fibers and fragmentation of the cardiac muscle fibers (head arrow) (H & E, ×400).

### Conclusion

The use of nanotechnology in medicine is expected to spread quickly. This study assessed the effects of nanocurcumin on oxidative stress and histopathological evaluation in an *in ovo* model. We show that nanocurcumin at a concentration of 100 ppm significantly decreases TG and enhances HDL-c in blood serum, and

therefore has potential to be conducive for cardiovascular disease prevention. However, a higher dose (1000 ppm) could be degenerative.

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