

## Nicarbazin Induce Oxidative Response and Colony Stimulating Factor Production in Mouse lung Cells In Vitro

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Nicarbazin is an effective and wide spread recognized substance for controlling the protozoal diseases with low number of short term adverse reactions. There are some molecular mechanisms proposed for its adverse effect but no one has conducted a research in vivo. Also there is no evidence found which can illustrate the long term adverse effects of the drug. It may hypothesized that Nicarbazin can affect the oxidative situation of cells by lipoprotein lipase activation. This study investigated the relationship between using Nicarbazin or its ingredients and oxidative stress situation by monitoring changes in the oxidative enzymes activity: Catalase, Glutathione peroxidase, Superoxide dismutase and specific biomarkers of oxidation: Dityrosine and Malondialdehyde. The study also investigates Nicarbazin's effects on colony stimulating factors in lung cells. In conclusion it found that Nicarbazin can worsen oxidative stress and increase colony stimulating factors which may have affect on long term adverse reactions of drug.

**Key words:** Nicarbazin, Oxidative Response, Oxidative enzyme,  
Oxidative biomarker, Colony Stimulating Factor.

It has been over 50 years since the first usage of Nicarbazin (NCZ) in veterinary practice and until today it has been the most widely recognized drug for controlling the broad-spectrum protozoal disease coccidiosis in industry<sup>1</sup>. In the United States, for starter feeds, 125 ppm of NCZ or 80 ppm in combination with other drugs is commonly used as an effective anticoccidial program<sup>2-4</sup>. Despite the powerful action and low resistance<sup>5</sup>, some adverse effects on broiler industry were detected recently. The main adverse reaction

is the effects on avian reproduction by reducing egg production, egg weight, and hatchability<sup>4</sup>. Another adverse effect associated is spiking mortality syndrome which is believed to have an unknown molecular mechanism<sup>6</sup>. Nicarbazin crystals consist of two different component: 4,4'-dinitrocarbanilide(DNC) which is the active ingredient and 2-hydroxy-4,6-dimethylpyrimidine (HDP) which helps DNC to absorb more in poultry GI tract and improved anticoccidial activity of Nicarbazin<sup>7</sup>.

The molecular mechanism of Nicarbazin is based on inhibiting the development of first and second generations'schizont stage of parasites. There are some molecular mechanisms proposed for Nicarbazin's avian adverse effect but no one has conducted a research in vivo. It has been demonstrated that Nicarbazin can increase the

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activity of lipoprotein Lipase. This may cause premature degradation of very low density lipoproteins and reduction in deposition of lipid in yolk. So the overall egg production and egg weight may decrease<sup>8-10</sup>. It has been demonstrated that NCZ can act as a carrier ionophore. Nicarbazin can enter to vitelline membrane and make it permeable. Damages caused by this disruption leads to egg yolk mottling and reduction in egg hatchability<sup>11-12</sup>.

Farm managers or workers exposed to NCZ through oral, dermal or inhalation route<sup>13</sup>. Mammalian toxicity studies (in maximum of two year) prove that Nicarbazin has no adverse effects except some fetotoxicities among which are delayed ossification and reduced fetal weight<sup>14</sup>. Unfortunately, by the best of literature review, no evidence was found which can illustrate the long term effects of this drug. But if we consider that NCZ can activate lipoprotein lipase<sup>8</sup>, and take into account the relationships between lipoprotein lipase expression and oxidative stress, concluded from different studies<sup>15-22</sup>, it may hypothesized that Nicarbazin can affect the oxidative situation of cells.

Reactive oxygen species (ROS) like Hydrogen peroxide and oxygen free radicals are the highly toxic chemicals in body<sup>23</sup>. Antioxidant system is developed to protect the cellular molecules from overproduction of these chemicals. catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) are three enzymes which this system has evolved, in order to defend the oxidative injury<sup>24</sup>. If these enzymes activities change abnormally, high toxic situation happens in cells and ROS attack membrane lipids, inactive metabolic enzymes and damage the nucleic acids leading to cell death<sup>25</sup>. By this chemical modification and cell damages many pathogenesis, disorder and chronic disease may occurs subsequently<sup>26</sup>. This study conducted to investigate the hypothesis that if there is any relationship in using Nicarbazin or its ingredients in worsening oxidative stress. The changes in oxidative status will be monitored by oxidative enzyme activity and specific biomarker of oxidation, Dityrosine(DT) and Malondialdehyde (MDA) which correlate the level of oxidative stress<sup>27</sup>. The study also aimed to reveal whether Nicarbazin can affect colony stimulating factors in lung cells.

## MATERIALS AND METHODS

### Chemicals

Nicarbazin (NCZ), 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) were purchased from Sigma Chemical Company (St. Louis, USA). Dulbecco's Modified Eagle Medium (DMEM) as Culture medium was obtained from Gibco Laboratories (Paisley, Scotland). Culture media solutions were sterilized by filtration through a 0.22- $\mu$ m filter (Acrodisc, Gelman). Catalase (E.C. 1.11.1.6), Glutathione peroxidase (EC 1.11.1.9), Superoxide dismutase, O,O'-dityrosine (DT), malondialdehyde (MDA) were obtained from sigma-Aldrich, Germany.

### Cell culture

Lungs and bone marrow of either sex Balb/c mouse (25-30 g; Pasteur institute, Iran) approximate 4-6 weeks old were maintained for the experiment. The lungs selected as experiment tissue because they are the most aerobic organ in body so they will be the most affected organ throughout the oxygen toxicity and ROS attack. Also it is studied that lung tissue are very rich source of CSF<sup>28</sup>.

DMEM applied as medium supplemented with six different concentrations of NCZ, HDP and DNC. Concentrations were of 2.5, 5, 10, 20, 30 and 40  $\mu$ M. Mice anesthetized by Ether. Lung conditioned medium used for primary culture cell. In sterile condition, their lungs removed and washed with normal saline. The lungs minced first and then incubated in 5 mL of each processed mediums for 48 hours at 37p C and prepared for measuring biomarkers.

For CSF evaluation, the whole culture centrifuged and supernatant preserved in 25p C as sample for next step. For other assays the sediments homogenizes in 50mM phosphate buffer (pH=7.0), centrifuged (20000g, 15min) and the supernatant used as sample for next steps.

### Catalase assay

Catalase (E.C. 1.11.1.6) activity was determined by spectrophotometric method of Abei based on the consumption of H<sub>2</sub>O<sub>2</sub>.<sup>(29)</sup> The assay reaction consists of 100mM phosphate buffer (pH=7.0), 15mM H<sub>2</sub>O<sub>2</sub> and 50 mL of sample in total volume of 3 mL. The reaction carried out in 30 seconds. H<sub>2</sub>O<sub>2</sub> consumption recorded by the changes in the rate of absorbance ( $\Delta A/min$ ) at 240nm by spectrophotometer, model Cintra 6

GBC (GBC Scientific Equipment, Australia). CAT activities were calculated using the molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> of 0.0394 mM<sup>-1</sup>cm<sup>-1</sup>. CAT activity of the sample was expressed as CAT units per milligram of protein.

#### Glutathione peroxidase assay

Glutathione peroxidase (EC 1.11.1.9) activity was determined by the method of Hopkins & Tudhope, based on oxidation of NADPH. (30) The assay reaction consist of 50mM phosphate buffer (pH=7.0), 2mM EDTA, 0.28 mM NADPH, 0.13mM GSH (Reduced Glutathione), 0.16U Glutathione reductase, 0.073 mM t-butyl hydroperoxide and 50mg protein. GPX activity of the sample was expressed as GPX units per milligram of protein.

#### Superoxide dismutase assay

Superoxide dismutase (EC 1.15.1.1) activity was determined by the method of Masayasu & Hiroshi, based on inhibition of Nitrobluetetrazolium (NBT) reduction in LC50. (31, 32) The assay reaction consist of 50mM Sodium Tris-cacodylic buffer (pH=8.2), 0.1mM EDTA, Triton X-100 1.42%, 0.055 mM NBT, 16mM Pirogalo land 50mg protein. SOD activity of the sample was expressed as SOD units per milligram of protein.

#### Dityrosine Measurement

O,O'-dityrosine was identified by reversed-phase HPLC with simultaneous UV-detection (280 nm) and fluorescence-detection. A phenomenex inertsil ODS 2 (150\_4.6 mm, 5 mm) HPLC column (Bester, Netherlands) equipped with a guard column was used for analyses. A gradient was formed by 10 mM ammonium acetate (pH=4.5 with acetic acid, and methanol) in 30 min. Flow rate was 0.8 mL/min. A standard solution of dityrosine was prepared according to the method of Amado *et al.*,<sup>33</sup>.

#### Malondialdehyde measurement

Proteins in sample were precipitated from lysed cells with 40% (w/v) TCA. In this process one molecule of MDA can precipitate with two molecules of TBA. This chromogen can absorb UV and will be analyzed by HPLC according to the method of Bird *et al.* (34) The chromogen was injected on Supelcosil (LC-18, 5 mm) reversed-phase HPLC column. (30×4.6mm<sup>2</sup>) Mobile phase consisted of 15% methanol in double distilled water degassed which was filtered through a 0.5mm filter

(Milipore, US). Flow rate was 2 mL/min.

#### CSF evaluation

Preserving 48h in 25°C, Samples were dialyzed over 24 h to remove excess of chemicals and maintain large molecules. The dialyzed sample were kept in hot bath water at 56°C for 30min and then centrifuged to receive CSF molecules. CSFs finally assayed on bone marrow culture and colonies were counted then.

#### Statistical methods

Each experiment was preformed triplicate. Descriptive statistics presented by means ± SD. The statistical significance of the results obtained for the groups was determined by ANOVA. P value < 0.05 was considered statistically significant for all experiments.

## RESULTS

For investigating the hypothesis that if Nicarbazin (or its ingredients) can worsen the oxidative stress, the oxidative status monitored by CAT, GPX and SOD (oxidative enzymes) activity assay and DT and MDA (specific oxidative biomarker) activity assay in 2.5, 5, 10, 20, 30 and 40 µM of NCZ, DNC and HPD respectively. It has been showed that NCZ, DNC and HPD can significantly increase the level of CAT and SOD in many dosages but they don't have any effect on the level of GPX. It has been showed that the substances also can significantly raise the level of DT and MDA in some dosages.

Table 1 demonstrates the alternations of oxidative enzymes and specific oxidative biomarkers level through enhancing the dosage of Nicarbazin. Statistical analysis shows that NCZ can significantly increase the level of CAT at 20 µM and higher, SOD at 2.5 µM and higher (except at 10µM), DT at 30 µM and MDA at 20 µM and higher in culture cells. But NCZ did not have any significant effect on increasing the level of GPX. Table 2 reveal the levels of markers through different dosages of 4,4,2-dinitrocarbanilide. Statistical analysis disclosed that DNC can significantly raise the level of CAT at 5 µM and higher (except at 10µM), SOD at 2.5µM, 30 µM and 40 µM, DT at 30 µM and higher and MDA at 5 µM and higher when compared with treated cultures in the absence of DNC. But DNC did not have any significant effect on raising the level of GPX.

Table 3 illustrate the elevation of enzymes and biomarkers levels by supplementing 2-hydroxy-4,6-dimethylpyrimidine dosage. ANOVA analysis

demonstrate that HDP can significantly increase the level of CAT at 2.5  $\mu$ M and higher, SOD at 30 $\mu$ M and 40  $\mu$ M, DT and MDA at 10  $\mu$ M and higher with

**Table 1.** Oxidative enzymes' and oxidative specific biomarkers' level through different dosage of Nicarbazin. Data are presented as means  $\pm$  SD

Concentration ( $\mu$ M)	Nicarbazin (NCZ)				
	CAT	GPX	SOD	DT	MDA
0	39.8 $\pm$ 3.9	21.2 $\pm$ 4.1	14.5 $\pm$ 2.6	23.4 $\pm$ 1.6	49.2 $\pm$ 6.3
2.5	36.7 $\pm$ 3.8	19.1 $\pm$ 3.1	*20.7 $\pm$ 2.3	27.5 $\pm$ 4.6	45.2 $\pm$ 7.1
5	41.4 $\pm$ 6.4	19.6 $\pm$ 3.3	*30.9 $\pm$ 6.0	24.2 $\pm$ 2.7	46.4 $\pm$ 9.4
10	38.1 $\pm$ 6.6	22.0 $\pm$ 4.8	22.7 $\pm$ 4.9	21.5 $\pm$ 2.0	49.5 $\pm$ 4.4
20	*23.6 $\pm$ 3.0	23.4 $\pm$ 4.5	*19.1 $\pm$ 4.6	24.5 $\pm$ 4.0	*62.3 $\pm$ 1.7
30	*13.6 $\pm$ 1.2	17.5 $\pm$ 3.0	*12.4 $\pm$ 1.8	*33.7 $\pm$ 6.7	*62.1 $\pm$ 6.9
40	*8.0 $\pm$ 1.0	15.8 $\pm$ 1.6	*11.3 $\pm$ 1.7	25.6 $\pm$ 3.5	*66.1 $\pm$ 6.1

\*Dosages which Nicarbazin significantly increase the level of Catalase, Glutathione peroxidase, Superoxide dismutase, Dityrosine and Malondialdehyde. (A p value < 0.05 was considered statistically significant)

**Table 2.** Oxidative enzymes' and oxidative specific biomarkers' level through different dosage of 4,4'-dinitrocarbanilide(DNC), an ingredient of Nicarbazin. Data are presented as means  $\pm$  SD.

Concentration ( $\mu$ M)	4,4'-dinitrocarbanilide(DNC)				
	CAT	GPX	SOD	DT	MDA
0	39.8 $\pm$ 3.9	21.1 $\pm$ 4.1	14.5 $\pm$ 2.6	23.4 $\pm$ 1.6	49.2 $\pm$ 6.3
2.5	45.4 $\pm$ 5.5	24.1 $\pm$ 4.6	*23.9 $\pm$ 4.8	26.0 $\pm$ 3.4	50.8 $\pm$ 7.0
5	*55.1 $\pm$ 7.6	22.8 $\pm$ 2.6	18.1 $\pm$ 5.7	20.8 $\pm$ 1.9	*36.7 $\pm$ 4.3
10	44.0 $\pm$ 4.9	21.7 $\pm$ 4.4	21.2 $\pm$ 5.2	27.3 $\pm$ 4.6	*38.1 $\pm$ 3.6
20	*21.3 $\pm$ 2.9	18.5 $\pm$ 3.1	16.8 $\pm$ 5.4	28.1 $\pm$ 5.1	*63.4 $\pm$ 6.1
30	*15.4 $\pm$ 4.5	15.6 $\pm$ 3.4	*9.6 $\pm$ 1.0	*37.5 $\pm$ 9.1	*66.6 $\pm$ 6.3
40	*9.4 $\pm$ 0.7	14.9 $\pm$ 2.4	*5.6 $\pm$ 0.9	*40.3 $\pm$ 8.4	*82.0 $\pm$ 6.1

\*Dosages which 4,4'-dinitrocarbanilide significantly increase the level of Catalase, Glutathione peroxidase, Superoxide dismutase, Dityrosine and Malondialdehyde. (A p value < 0.05 was considered statistically significant)

**Table 3.** Oxidative enzymes' and oxidative specific biomarkers' level through different dosage of 2-hydroxy-4,6-dimethylpyrimidine (HDP), an ingredient of Nicarbazin. Data are presented as means  $\pm$  SD

Concentration ( $\mu$ M)	2-hydroxy-4,6-dimethylpyrimidine (HDP)				
	CAT	GPX	SOD	DT	MDA
0	39.8 $\pm$ 3.9	21.1 $\pm$ 4.1	14.5 $\pm$ 2.6	23.4 $\pm$ 1.6	49.2 $\pm$ 6.3
2.5	*50.5 $\pm$ 3.2	22.0 $\pm$ 5.1	15.0 $\pm$ 4.4	21.9 $\pm$ 4.4	46.5 $\pm$ 6.4
5	*57.8 $\pm$ 3.9	19.1 $\pm$ 4.3	15.0 $\pm$ 1.6	19.0 $\pm$ 5.7	43.4 $\pm$ 6.2
10	*58.6 $\pm$ 3.8	21.3 $\pm$ 3.4	12.0 $\pm$ 1.6	*14.4 $\pm$ 2.5	*35.6 $\pm$ 5.0
20	*68.0 $\pm$ 5.1	22.4 $\pm$ 3.8	15.2 $\pm$ 3.2	*11.3 $\pm$ 2.3	*31.4 $\pm$ 4.0
30	*68.0 $\pm$ 5.1	23.9 $\pm$ 5.1	*22.4 $\pm$ 2.8	*13.9 $\pm$ 4.1	*25.0 $\pm$ 5.6
40	*64.9 $\pm$ 5.2	22.8 $\pm$ 5.3	*24.0 $\pm$ 1.7	*9.5 $\pm$ 2.2	*22.9 $\pm$ 4.9

\*Dosages which 2-hydroxy-4,6-dimethylpyrimidine significantly increase the level of Catalase, Glutathione peroxidase, Superoxide dismutase, Dityrosine and Malondialdehyde. (A p value < 0.05 was considered statistically significant)

**Table 4.** The effect of Nicarbazin and its ingredients on the level of Colony Stimulating Factor (CSF) in vitro. Data are presented as means  $\pm$  SD.

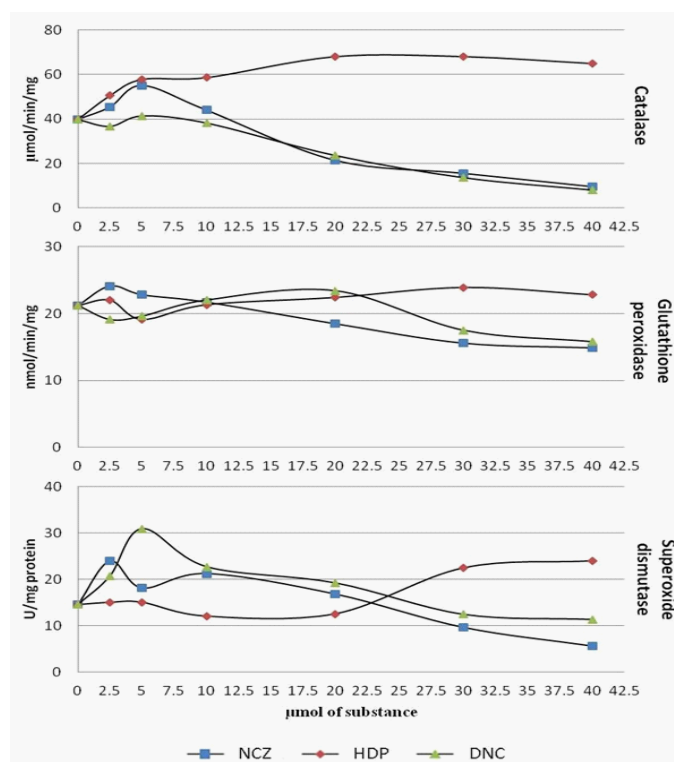
Concentration ( $\mu$ M)	Colony Stimulating Factor (CSF)		
	NCZ	DNC	HDP
0	58.0 $\pm$ 6.6	58.0 $\pm$ 6.6	58.0 $\pm$ 6.6
2.5	66.7 $\pm$ 7.1	59.3 $\pm$ 7.5	58.7 $\pm$ 6.6
5	*72.3 $\pm$ 7.6	*86.3 $\pm$ 11.6	64.7 $\pm$ 8.6
10	54.0 $\pm$ 4.6	*77.0 $\pm$ 9.2	55.3 $\pm$ 7.6
20	*44.0 $\pm$ 4.6	*38.3 $\pm$ 6.7	59.7 $\pm$ 6.0
30	*26.0 $\pm$ 8.2	*16.3 $\pm$ 5.7	*86.3 $\pm$ 8.0
40	*22.3 $\pm$ 8.8	*0	*83.7 $\pm$ 5.0

\*Dosages which Nicarbazin, 4,4'-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine significantly increase the number of CSF colonies. (A p value  $< 0.05$  was considered statistically significant)

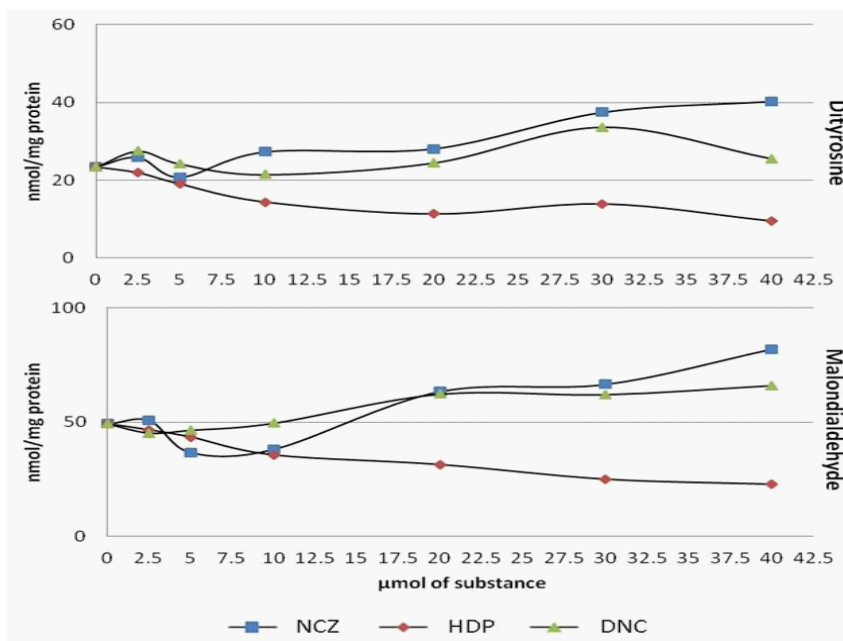
respect to control. Like NCZ and DNC, the HDP did not have any significant effect on increasing the level of GPX.

For investigating the hypothesis that if Nicarbazin (or its ingredients) can affect colony stimulating factors in mouse lung cells, different

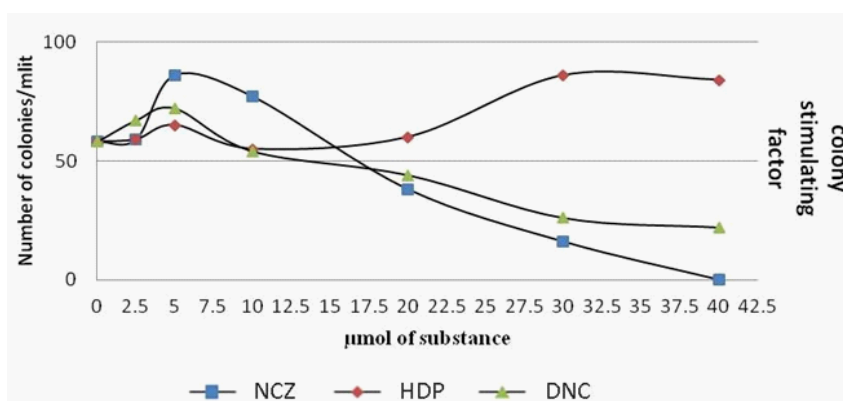
dosage of NCZ, DNC and HPD added to processed medium. Table 4 demonstrates the results of CSF colony counting in different concentration of substances. As it can be seen NCZ and DNC elevate the count of CSF through 5  $\mu$ M but higher dosages progressively diminished the level of CSF. In respect



**Fig. 1.** Effect of Nicarbazin (NCZ) and its ingredients, 4,4'-dinitrocarbanilide(DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) on oxidative enzymes activity: Catalase (CAT), Glutathione peroxidase (GPX) and Superoxide dismutase (SOD)



**Fig. 2.** Effect of Nicarbazine (NCZ) and its ingredients, 4,4'-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) on specific biomarker of oxidation: Dityrosine(DT) and Malondialdehyde (MDA)



**Fig. 3.** Effect of Nicarbazine (NCZ) and its ingredients, 4,4'-dinitrocarbanilide(DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP) on colony stimulating factors in lung cells

to control Nicarbazine and 4,4'-dinitrocarbanilide can significantly influenced CSF production at 5  $\mu$ M and higher(Nicarbazine can not increase the number of colonies at 10  $\mu$ M). It has been showed that in 40  $\mu$ M of DNC, colony production completely inhibited any no colony existed anymore. 2-hydroxy-4,6-dimethylpyrimidine also can elevate the number of colonies at 30  $\mu$ M and 40  $\mu$ M. Table 4 numbered the dosages which Nicarbazine, 4,4'-dinitrocarbanilide and 2-hydroxy-

4,6-dimethylpyrimidine significantly increase the number of colonies of colony stimulating factors.

## DISCUSSION

Antioxidant system protects body from the imbalance of superoxide radicals ( $O_2^-$ ), hydrogen peroxides ( $H_2O_2$ ), hydroxyl radicals ( $HO$ ) and singlet oxygen ( $^1O_2$ ) which are the byproducts of biological metabolism. CAT, SOD and GPX

are three enzymes of this system developed to defense this harmful ROS attack and oxidative injury<sup>24</sup>. This study investigated the hypothesis of worsening oxidative stress and increasing of colony stimulating factors from using Nicarbazin to clarify the molecular mechanism of this drug and conduct other studies to disclose the drug's medical reactions and longtime adverse effects.

The results of this study demonstrate the effect of Nicarbazin and its ingredients on oxidative enzymes activity. Figure 1 illustrates the effect of NCZ, HPD and DNC on CAT, SOD and GPX. For definite characterization of bulk protein damage by ROS attack, specific biomarkers of oxidation have been evaluated. Level of Dityrosine measured because this molecule appears when hydroxyl radical cross-links 2 tyrosines or is generated by tyrosyl radicals<sup>27</sup>. Also MDA were evaluated for demonstrating the lipid peroxidation by ROS attack<sup>34</sup>. Figure 2 illustrates the effect of NCZ, HPD and DNC on DT and MDA. These figures show that Nicarbazin and its ingredients can significantly raise the biomarkers level in most dosages like many other xenobiotics or exogenous chemicals<sup>35</sup>. Figure 2 represent a base line level of DT and MDA in cells which is the evidence for bulk cell proteins and lipids oxidation damage in normal cells. Elevating in the level of biomarkers is an evidence for calling these substances as sources for oxidative stress. Figure 1 also demonstrates that lung cells will elevate their antioxidative system status by antioxidant enzymes over expression to defense these sources.

It can be highlighted that the mechanism of action of Nicarbazin and its ingredients in alternation of oxidative stress level may have different pathway because there are lots of structures proposed but none is precisely approved. As it said NCZ can increase the action of some enzymes in the metabolism of body, like lipoprotein lipase<sup>8</sup>, and variation in the activity of these enzymes can consequently impress the antioxidative defense system<sup>15-22</sup>. In conclusion lipoprotein lipase activation may be the most effective pathway in calling Nicarbazin as sources for oxidative stress.

Fig. 3 illustrates the effect of NCZ, HPD and DNC on the number of colony stimulating factors in lung cells. Colony stimulating factors are one of the most important molecules which

affect the oxidative stress status<sup>36-39</sup>. In conclusion, Nicarbazin may raise the number of colony stimulating factors by elevating this defense mechanism in cells. It is recommended that in future, other studies will investigate pathogenesis, disorder and chronic disease by using Nicarbazin (current or toxic doses) which may occur by these pathways in body.

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