

## Molecular mechanism of Copper Albumin Complex on NDEA induced brain vascular damage via promoting VEGF expression

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### Abstract

Nitrosodiethylamine (NDEA) is a potent oxidant induces neurodegeneration via (reactive oxygen species) ROS. Copper is an important metal essential for scavenging free radicals, development of central nervous system (CNS) and redox angiogenesis signaling. Vascular endothelial growth factor (VEGF) is well known as efficacious and long-term signal that stimulates angiogenesis, where its expression is copper dependent. We examined the copper protective effect against brain vascular damage initiated by NDEA. NDEA induces brain vascular wall damage, necrosis with interstitial hemorrhage and diminishes VEGF expression. Histopathological examination showing a great improvement of brain tissue in copper treated mice with significant increase in VEGF expression. Higher levels of intracellular copper can stimulate angiogenesis and exhibited a significant protection against NDEA induced brain vascular damage, confirming its ability to enhance antioxidant activity and angiogenesis initiation. Our report presents first evidence that inducible VEGF expression in brain is sensitive to copper; moreover, copper-based therapeutics represents a novel approach to reduce brain vascular damage induced by NDEA generated ROS.

### Keywords:

Copper, NDEA, Nitrosamines, ROS, VEGF.

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**Competing interest:** The authors have declared that no competing interest exists.



## Introduction

Nitrosamines is a wide class of chemical oxidant and carcinogens which are present in large number of food stuff such as dried milk, cheese, sausage, smoked fish, processed meat, alcoholic beverages and tobacco smoke. They are formed in acidic condition of stomach from nitrate and amines which are commonly found as residues of agriculture chemicals, pharmaceutical drugs, food constituent and additives (Lijinsky, 1999; Mittal et al., 2006). The toxic and mutagenic effects of nitrosamines are attributed to alkylation N-7 of guanine, leading to destabilization and increased breakage of DNA (Bansal et al., 2005). Moreover, reactive oxygen species (ROS) generated from Activated nitrosamines increase oxidative stress, lipid peroxidation, DNA damage and protein adduct formation (Balamurugan and Karthikeyan, 2012). DNA damage and Oxidative stress activate pro-inflammatory cytokines and promote insulin resistance, both of which are key elements in the pathogenesis of Alzheimer's disease (AD), and experimental models of AD-type neurodegeneration (de la monde and tong, 2009). Copper is one of the essential trace metals that can easily switch from oxidized ( $\text{Cu}^{\text{II}}$ ) to the reduced form ( $\text{Cu}^{\text{I}}$ ) in biological media. This character makes copper important redox catalyst for many essential processes including energy production, collagen synthesis, antioxidant activity via removal of free radicals, degradation and biosynthesis of neurotransmitter and redox signaling in angiogenesis (Duncan and white, 2012; Hatori and Lutsenko, 2016). In addition copper is important for development and function of central nervous system (CNS), copper exert considerable control over synaptic transmission via binding to and modulation the function of  $\gamma$ - amino butyric acid type A (GABAA) receptors, N-methyl-D-aspartate (NMDA) receptors and voltage-gated  $\text{Ca}^{2+}$  channels contributes (Gaier et al., 2013). Due to physiological importance of

copper different copper complexes were synthesized and investigated for therapeutic uses. Angiogenesis, the formation and maintaining of new capillaries from existing vasculature, is necessary for physiological function of tissues as well as progression of diseases such as inflammation and cancer (Risau, 1997; Hanahan and Folkman, 1996). Variable signaling molecules, such as vascular endothelial growth factor-vascular endothelial growth factor receptor (VEGF-VEGFRs), angiopoietin-Tie, ephrin-Eph receptors and the Delta-Notch system play important roles in angiogenesis (Ferrara and Kerbel, 2005). VEGFs, on the other hand, have pro-angiogenic capability for maintaining physiological levels of diverse tissues and for the development of new blood vessels to overcome ischemic diseases (Shibuya, 2011). Inducible VEGF also has a direct influence on neural cell. Abnormal regulation expression of VEGF has now been implicated in several neurodegenerative disorders, including moto-neuronl degeneration (Storkebaum and Carmeliet, 2004). The VEGF-VEGFR system is an intriguing target for pro-angiogenic therapy in the treatment of neuronal degeneration and ischemic diseases, as well as an essential target for anti-angiogenic therapy in cancer (Shibuya, 2011). Copper has been found to stimulate factors involved in micro vessel formation and development among which VEGF (Xie and Kang, 2009). The present study was designed to evaluate the chemo-preventive effect of copper albumin complex against vascular damage in brain of mice intoxicated by nitrosodiethylamine (NDEA).

## Materials and methods

### Reagents:

#### Nitrosodiethylamine (NDEA):

NDEA (CAS NO, 55-18-5) was purchased from Sigma Aldrich Company

for Trading Chemicals, Medicines and Medical Appliances, Egypt.

### **Copper albumin complex:**

Copper-albumin complex was prepared by Dr. Ahmed Y. Nassar, Professor of Biochemistry, Faculty of Medicine, Asyut University, Asyut, Egypt (Elgazzar et al., 2012). The copper albumin complex was suspended in water and used as a prophylactic agent orally at a dose (817µg/kg B.W).

### **Experimental animals:**

In this study, forty white male albino mice, aged 4-5 weeks and weighing 25 gm, were used in the experimental investigation. Mice were obtained from the Laboratory animal research center, Faculty of veterinary medicine, Asyut University. Mice were accommodated in appropriate normal conditions. Before the beginning of the experiment, animals were left 7 days for acclimatization. Animals were admitted in the Department of Biochemistry, Faculty of Veterinary Medicine, South Valley University following the ethical consideration of experimental animals of South Valley University (No. 32/14-03-2022).

### **Experimental design:**

The animals were randomly divided into four groups of ten animals each;

**A) Group I (normal control group):** Mice were given water orally without any treatment.

**B) Group II (normal treated group):** Mice were given oral administration of copper albumin complex at a dose of (817µg/kg B.W three times weekly) in the morning from 2<sup>nd</sup> week till the end of experiment (Elgazzar et al., 2012).

**C) Group III (carcinogen control group):** Mice were given N-Nitrosodiethylamine (NDEA) at a dose of (50mg/kg body weight i.p) once a week for six weeks; beginning

on the second week. NDEA was freshly prepared in normal saline (Singh et al., 2009; Da Costa et al., 2014; Younis et al., 2019).

**D) Group IV (prophylactic group):** From the second week to the end of the experiment, mice were given oral administration of copper albumin complex at a dose of (817g/kg B.W three times weekly). In addition, mice were given N-Nitrosodiethylamine (NDEA) at a dose of (50mg/kg body weight i.p) weekly for 6 weeks, starting on the second week. Mice were pretreated with copper albumin complex in the morning and then injected with NDEA 6 hours later. This new technique was applied to enhance antioxidant defense mechanism before toxin administration.

Mice in each group were scarified by cervical decapitation at the end of the experiment on the 13th week, and brain tissues from the same mouse were quickly dissected and separated into two portions. One part was kept in formalin for histopathological analysis. For RNA/protein isolation other fresh tissue parts were kept at -80 °C.

### **RT-PCR:**

QIAzol Reagent (QIAGEN®, QIAzol™) was used to extract total RNA according to the manufacturer's instructions. A nanodrop ND-1000 spectrophotometer was used to check total RNA concentration and quality. The RNA quality was estimated by the 260/280 nm absorbance ratio.

The cDNA was synthesized from 1 µg RNA with the RNA PCR kit with oligo(dT) primers (TaKaRa) and was then used as a template for rt-PCR analysis. Band intensities were quantified with the NIH Image J software. Primer sequences were used (Nam et al., 2010) and β-actin primer was manually designed.

Primer	Forward (from 5' to 3')	Reverse (from 5' to 3')
VEGF	CAGGCTGCTCTAACGATGAA	CAGGAATCCCAGAAACAACC
$\beta$ -actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC

### Histopathology analysis

Specimens from brain tissues were fixed in a 10% buffered formalin solution, then exposed to various concentrations of ethyl alcohol (70%, 80%, 90%, and 100%) and embedded in paraffin blocks before being cut into 2-m-thick sections. Paraffinized sections were deparaffinized with xylene and rehydrated through a decreasing gradient of ethanol solution. Slides were stained with hematoxylin and eosin (H&E), cover slipped with mounting medium, and viewed under a light microscope. Slides were scanned with digital scanner (MIRAX; Carl Zeiss) and then viewed with MIRAX software (Carl Zeiss).

### Statistical analysis

The two-tailed Student's t-test and Mann-Whitney U-test for comparisons within each parameter, while ANOVA and Dunnett t-tests were used for multiple comparisons; compare each value of

treatments with a single control. Differences considered statistically significant when the P value was <0.01.

### Results

#### 1- The mRNA expression of VEGF in brain tissue.

The mRNA expression of VEGF was detected by rt-PCR, where it is diminished significantly in NDEA treated group in comparison with the control group. Interestingly after co-treatment between NDEA and copper the mRNA expression of VEGF was significantly retrieved again as showed in Fig. 1(a). To confirm previous result, the band intensity of the expressed VEGF were measured by using Image J software and statistically checked showing the significant increase in the expression of VEGF mRNA as showed in Fig. 1(b), where the data are representative a three different individuals in each group as the P value was <0.01.

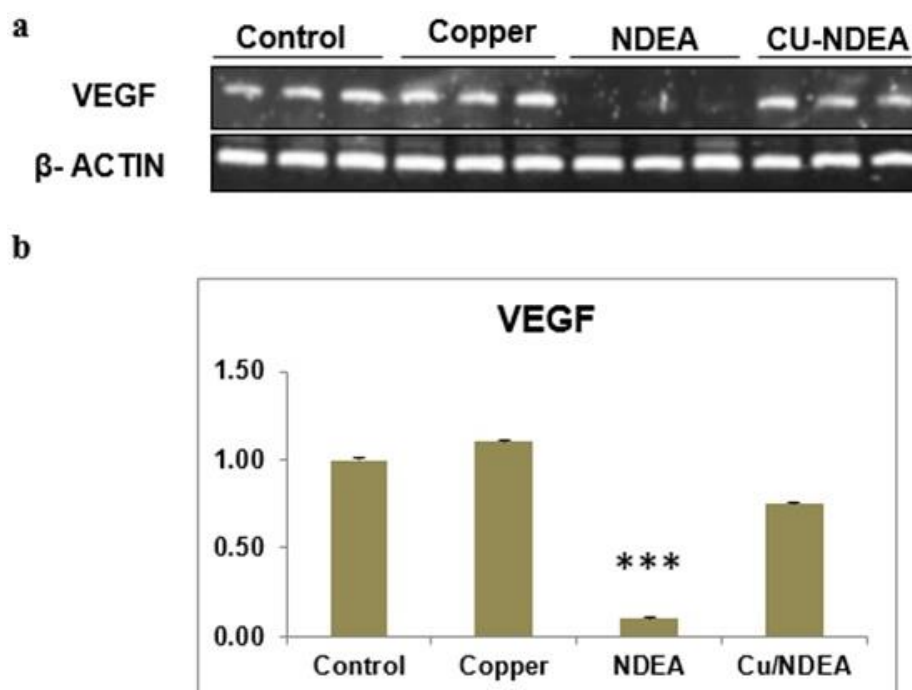
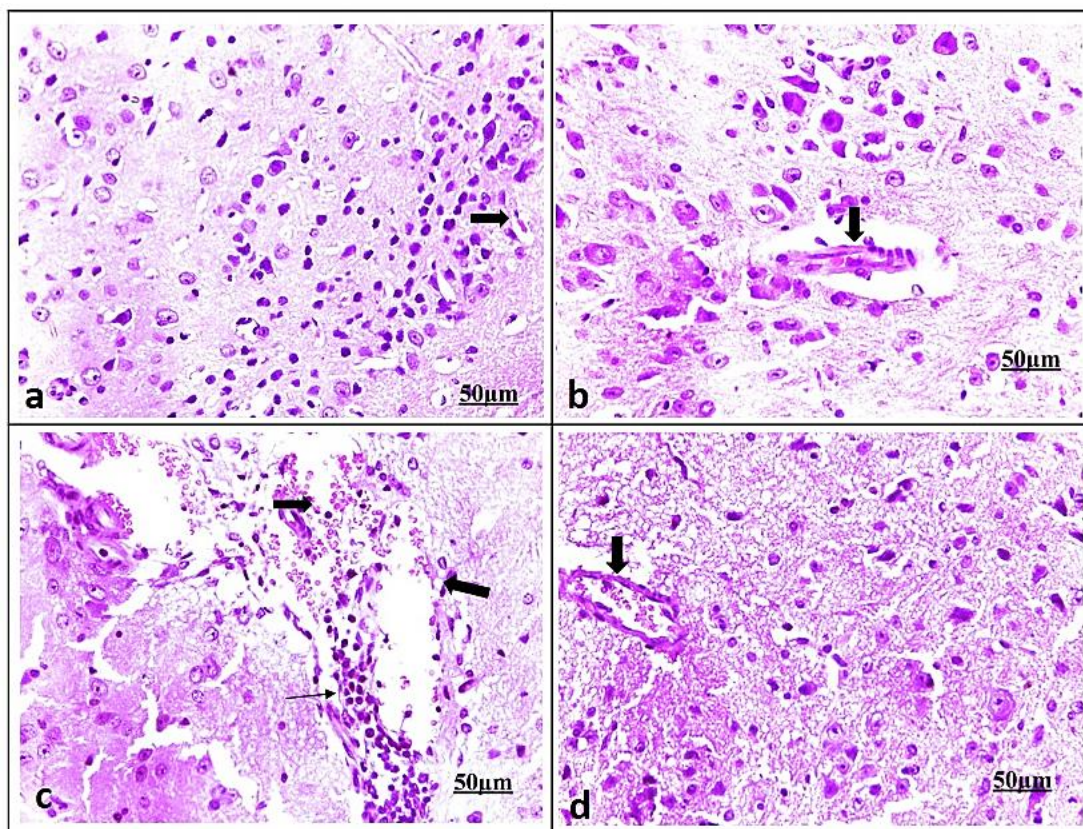


Fig. 1. The mRNA expression of VEGF in brain tissue

## 2- Histopathological changes in the vascular tissue in cerebrum of mice.

The tissue we stained by H&E, where the control group showed normal blood vessels as presented in Fig. 2(a). Furthermore, the copper treated group also showed normally appeared blood vessels with mild dilated Virchow robin space as in Fig. 2(b). Moreover, NDEA group showing

sever vascular damage represented by a sever necrosis in the wall of the blood vessels with interstitial hemorrhage as in Fig. 2(c). Furthermore, NDEA plus copper group showing mild congestion with intact endothelium in the brain blood vessels as in Fig. 2(d). Where it confirms the ability of copper complex to counteract the NDEA generated ROS adverse effect.



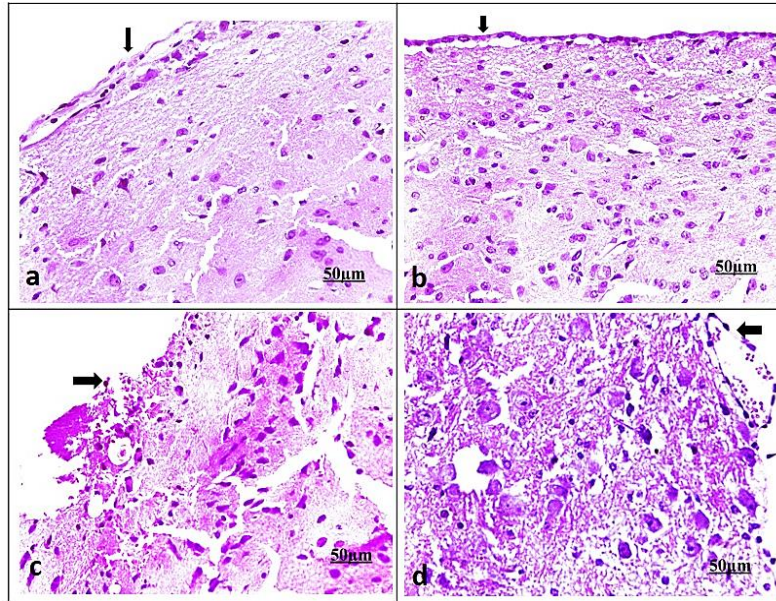
**Fig. 2. Histopathological changes in the vascular tissue in cerebrum of mice**

## 3-Histopathological changes in the vascular tissue in subarachnoid space of mice.

The tissue we stained by H&E, where the control group showed normal subarachnoid vasculature as presented in Fig. 3(a). Furthermore, the copper treated group also showed normally appeared blood vessels of subarachnoid tissue as in Fig. 3(b).

Moreover, NDEA group showing sever hemorrhage with sever necrosis in wall of the blood vessels of subarachnoid spaces as in Fig. 3(c). Furthermore, NDEA plus copper group showing Mild congestion with intact endothelium of wall of blood vessels as in Fig. 3(d).

Furthermore, histopathological scoring and features of brain tissue was investigated in the different groups, where the vascular necrosis, angiogenesis and interstitial hemorrhage were recorded as in Table 1.



**Fig. 3. Histopathological changes in the vascular tissue in subarachnoid space of mice.**

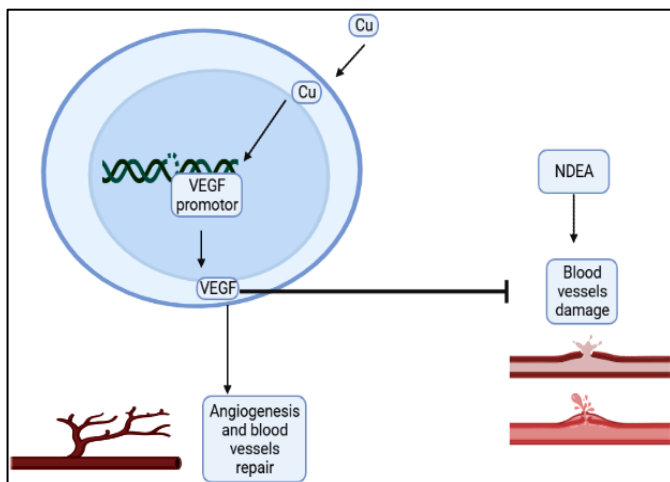
**Table 1: Histopathological feature of brain tissues**

Group	Pathological lesion	Necrosis of the blood vessels	Angiogenesis	Interstitial hemorrhage
Control		-ve	-ve	-ve
Copper		-ve	+++	-ve
NEDA		+++	+	+++
NEDA plus copper		+	++	+

-ve ... absent, +..... mild, ++... moderate, +++.....sever

**4- Graphical diagram showing the possible mechanism of copper albumin complex on NDEA generated ROS via enhancing inducible VEGF gene expression:**

Where copper enters to the nucleus and stimulate the VEGF promoter then enhance the expression of VEGF and stimulate angiogenesis as showed in Fig.



**Fig. 4. Graphical diagram showing the possible mechanism of copper albumin complex on NDEA generated ROS via enhancing inducible VEGF gene expression.**

## Discussion

This present work has been designed to determine the chemo-preventive role of copper albumin complex against toxic effect of nitrosodiethylamine (NDEA) on blood vessel of various parts in brain tissue of mice. The histopathological result of brain tissue in mice intoxicated with NDEA show severe necrosis in wall of blood vessel with interstitial hemorrhage. Previously reported that liver tissue treated with NDEA shows damage in hepatic tissue with severe hemorrhage and attributed the hepatic damage to elevated level of oxidative stress and decline level of antioxidant upon exposure to NDEA (Mukherjee and Ahmad, 2015). Our report showing that the group which treated with copper albumin complex show improvement in wall of blood vessel this may be attributed to antioxidant activity of copper. As confirmed by previous study that, copper complexes show prophylactic effect against many diseases and able to reduce the oxidative stress and elevated level of antioxidant enzymes (Elgazzar et al., 2012; Shatat et al., 2013).

Gene expression of vascular endothelial growth factor (VEGF) shows significant decrease in NDEA treated group than control. Recent study has been demonstrated that reduced VEGF level result in neurodegeneration in part by impairing perfusion of neural tissue. Moreover, reduced VEGF expression in mice via subtle targeted deletion of the hypoxia response element in the promoter of the VEGF gene (VEGF $\delta/\delta$ mice) cause adult-onset motoneuron degeneration (Oosthuysen et al., 2001). de la monte and Tong (2009) denoted that NDEA exposure cause neurodegeneration via elevated level of oxidative stress, lipid peroxidation, DNA damage, impairments in energy metabolism, acetylcholine homeostasis and

insulin/IGF signaling mechanisms (de la monte and Tong, 2009).

Our data represents significant increase in the expression of VEGF in mice treated by copper albumin together with NDEA compared to intoxicated group. As copper has been found to stimulate factors involved in micro vessel formation and development among which VEGF, which plays pivotal role in angiogenesis. Copper is required for activation of hypoxia inducible factor1 (HIF1), critical transcription factor that regulate expression of VEGF. Moreover, excess copper can inhibit HIF-1 $\alpha$  (the rate limiting component of HIF1) degradation and promote stabilization, thus leading to its accumulation in cytoplasm and its activation (Xie and Kang, 2009). In addition there is increasing evidence that blood vessels and angiogenic factors such as VEGF play an important role in control of neurogenesis (Black et al., 1990; Jin et al., 2002; Louissaint et al., 2002), also VEGF increased vascularization and reduced retrograde degeneration of transected cortico-spinal tract axons, as well as stimulating some axons to regenerate over the damaged area and this favorable effect of VEGF was returned to a vascular effect (i.e., restoration of neural blood flow), in addition a direct effect on Schwann cells (Schratzberger et al., 2000). Our data represents that, copper-based therapeutics represents a novel approach to reduce brain vascular damage induced by NDEA generated ROS and oxidative stress, enhancing our hypothesis about the novelty of using copper complexes as an antioxidant agent in the permissible doses to counteract the ROS induced damage along the molecular level.

## Conflict of interest

The authors declare that there is no conflict of interest.

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