Argyrophilic nucleolar organizer regions as a promising biomarker for the detection of brain hypoxia levels caused by different doses of carbon monoxide poisoning

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Abstract

Objectives: The purpose of the study is to investigate whether there is any relationship between mean argyrophilic nucleolar organizing regions (AgNOR) number and total AgNOR area/total nuclear area (TAA/TNA) ratio and the levels of brain hypoxia after exposure to different acute doses of carbon monoxide (CO) gas. **Methods:** Each experimental group was exposed to CO gas (concentrations of 1,000, 3,000 and 5,000 ppm). Then, the rats were anesthetized, and blood samples were taken from the right jugular vein for carboxyhemoglobin levels detection. The rats were sacrificed on seventh day. AgNOR staining was applied to brain tissues. TAA/TNA and mean AgNOR number were detected for each nucleus. **Results:** Significant differences were detected among all groups for TAA/TNA ratio, mean AgNOR number and carboxyhemoglobin level. According to a double comparison of groups, the differences between control and 1,000 ppm, control and 3,000 ppm, control and 5,000 ppm, and between 1,000 and 5,000 ppm were significant for TAA/TNA ratio. When mean AgNOR number was considered, significant differences were detected between control and 1,000 ppm, control and 3,000 ppm, control and 5,000 ppm, and between 1,000 and 3,000 ppm. **Conclusion:** AgNOR proteins may be used for early detection of the duration, intensity, and damage of brain injury caused by CO poisoning. Thus, effective treatment strategies can be developed for the prevention of hypoxic conditions.

KEY WORDS: Argyrophilic nucleolar organizer regions. Brain. CO poisoning. Nucleolar organizer regions. rDNA.

Regiones organizadoras nucleolares argirófilas como un biomarcador esperanzador para la detección de niveles de hipoxia cerebral causados por diferentes dosis de intoxicación de monóxido de carbono

Resumen

Objetivos: El objetivo del estudio es investigar si existe alguna relación entre el número medio de regiones organizadoras nucleolares argirófilas (AgNOR) y la proporción de área total de AgNOR/área nuclear total (TAA/TNA) y los niveles de hipoxia cerebral en la exposición a diferentes dosis agudas de gas monóxido de carbono (CO). **Métodos:** Cada grupo experimental fue expuesto a gas CO (concentraciones de 1,000, 3,000 y 5,000 ppm). Luego las ratas fueron anestesiadas, se tomaron muestras de sangre de la vena yugular derecha para la detección de los niveles de carboxihemoglobina. Las ratas se sacrificaron el séptimo día. Se aplicó tinción con AgNOR en los tejidos cerebrales. Se detectaron el TAA/TNA y el número medio de AgNOR para cada núcleo. **Resultados:** Se detectaron diferencias significativas entre todos los grupos para la relación TAA/TNA, el número medio de AgNOR y el nivel de carboxihemoglobina. Según la doble comparación de grupos, las

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diferencias entre control y 1,000 ppm, control y 3,000 ppm, control y 5,000 ppm y 1,000 y 5,000 ppm fueron significativas para la relación TAA/TNA. Cuando se consideró el número de AgNOR medio, se detectaron diferencias significativas entre control y 1,000ppm, control y 3,000ppm, control y 5,000 ppm y 1,000 y 3,000 ppm. **Conclusión:** Las proteínas AgNOR pueden usarse para la detección temprana de la duración, intensidad y daño de la lesión cerebral causada por la intoxicación por CO. Por lo tanto, se pueden desarrollar estrategias de tratamiento efectivas para la prevención de condiciones hipóxicas.

PALABRAS CLAVE: Regiones organizadoras nucleolares argirofílicas. Cerebro. Intoxicación por CO. Regiones organizadoras nucleolares. ADNr.

Introduction

Carbon monoxide (CO) gas is non-irritating, tasteless, odorless, colorless, and is formed as a result of incomplete combustion of carbon-containing substances. CO gas sources include fires, wildfires, faulty gas boilers, motor vehicle exhaust fumes, etc. There are 20,000-50,000 cases of CO-exposure poisoning in the US every year. CO poisoning is known as the cause of many fatal poisonings in some countries¹.

CO gas rapidly diffuses through alveolar capillary membranes, binds to oxygen and leads to the formation of carboxyhemoglobin (COHb). CO affinity for Hb is approximately 200-250 times higher than that of oxygen. CO poisoning causes hypoxia in the body, and the brain is one of the organs that is most sensitive to hypoxia. Hippocampal atrophy and changes in subcortical structures and globus pallidus have been observed^{1,2}.

Nucleolar organizer regions (NORs) are genetic loci on chromosomes. These regions are produced from proteins and ribosomal DNA (rDNA), with some corresponding to argyrophilic features. NORs are DNA loops, repeated in series with intergenic spacers and transcribed into ribosomal RNA (rRNA), which is part of mature ribosomes in nucleoli, and finally becomes part of mature ribosomes in the cytoplasm. NORs are functional subunits of the nucleolus³.

To the best of our knowledge, there are no studies on the relationship between CO poisoning and argyrophilic nucleolar organizer region-associated proteins (AgNORs) in brain tissues. AgNORs reveal the metabolic activity, proliferation and self-renewal potential of cells damaged in ischemic events. Therefore, our objective was to evaluate the potential effects of CO poisoning on brain tissue NOR protein synthesis and to investigate whether there is any relationship not only between the amount of AgNOR protein and COHb levels, but also between the degree of hypoxia and both AgNOR amounts (mean AgNOR number and total AgNOR area/total nuclear area ratio [TAA/TNA]) in current study.

Materials and methods

Experimental procedures

The present study was approved in accordance with internationally accepted principles for the care and use of laboratory animals by the local ethics committee for animal experiments. We used brain tissues of the rats that were employed in a previous study². Twenty adult male albino Wistar rats (between 150 and 180 g of weight) selected from the same breed were included in the study.

Four groups were formed, each one containing five rats as follows:

- Group A: control group (consisting of rats exposed to room air).
- Group B: formed by rats exposed to CO at low concentration (mixture of room air and 1,000 ppm of CO).
- Group C: formed by rats exposed to CO at medium concentration (mixture of room air and 3,000 ppm of CO).
- Group D: composed of rats exposed to CO at high concentration (mixture of room air and 5,000 ppm of CO).

Three steel tubes (Habas, Industrial and Medical Gases Production Industries Inc., Kocaeli, Turkey) containing 10 L of CO gas and different air mixture concentrations (1,000, 3,000 and 5,000 ppm) were used. All rats were exposed to CO gas in a closed plastic chamber (size: 20 x 40 x 60 cm³). There were two 2-cm diameter openings (outlet and inlet) on the plastic chamber opposite sides.

Groups B, C and D were exposed to CO gas at three densities (1,000 ppm, 3,000 ppm and 5,000 ppm) with a flow rate of 4 L/minute for 30 minutes in the plastic chamber. After seven days of exposure, the rats were anesthetized with 50 mg/kg of ketamine hydrochloride by intraperitoneal route (Ketalar, Parke-Davis, Eczacibasi, Istanbul-Turkey) and 3 mg/kg of xylazine hydrochloride by intraperitoneal route (Rompun, Bayer

AG, Leverkusen-Germany). Blood samples for the detection of COHb level were obtained from the right jugular vein by catheterization on each group. After the blood sample was taken, the catheter was removed.

Under anesthesia, the rats were placed in the supine position, the chest wall was shaved and disinfected using a 10% povidone iodine solution for the surgical procedure. Thoracotomy was performed through a midline incision. Fixation by perfusion was obtained with a 10% formaldehyde solution (administered by intracardiac route). Brain tissues were then excised and placed in a 10% formaldehyde solution for fixation. After that, tissues were embedded in paraffin blocks after the usual tissue processing protocols.

AgNOR proteins detection

4 μ m-thick sections of brain tissue were obtained, deparaffinized in xylene solution and rehydrated in graded alcohol solutions. The slides were then air-dried at room temperature for 15 minutes and subsequently fixed in absolute methanol for 5 minutes. AgNOR staining was applied interchangeably using a modified form of the Benn and Perle⁴ and Lindner⁵ protocols. The slides were evaluated using a light microscope (Olympus CHK 2, Olympus, Japan), and brain tissues were photographed with a digital camera (Axio Cam ICc5, ZEISS, Germany). The captured images were transferred to a digital image processing program (ImageJ 1.53f, National Institutes of Health, Bethesda, Maryland, USA). AgNOR area and nuclear area measurements were updated using the “free-hand selection” tool. Fifty nuclei were evaluated per rat and the TAA/TNA ratio and mean AgNOR number were detected for each brain cell nucleus.

Laboratory analysis

COHb levels were measured with blood gas analysis. The relationship between COHb levels and brain tissues was evaluated. Blood gas analysis was carried out using the ABL 800 device (Radiometer, Carlsbad, California, USA).

Statistical analysis

The Statistical Package for Social Sciences (SPSS), version 22, was used for statistical analysis. When the

study results were evaluated, in addition to descriptive statistics (number, mean, standard deviation, median and range), Kruskal-Wallis test was used for the comparison of all groups, whereas Mann-Whitney's U-test was resorted to for pairwise comparison of groups. In addition, a polynomial regression test was performed. A p-value < 0.05 was accepted as statistically significant.

Results

Mean AgNOR number and TAA/TNA ratio of each subgroup as well as AgNOR number and TAA/TNA ratio averages are shown in table 1. When all groups were considered, statistically significant differences were detected between all groups both for TAA/TNA ratio (χ^2 : 14.417; p = 0.002) and mean AgNOR number (χ^2 : 14.339; p = 0.002), respectively (Table 1 and Fig. 1). To understand these differences caused by the groups, a binary comparison was performed between them. According to group-wise double comparison for the TAA/TNA ratio, the differences between control and 1,000 ppm (Z: -2.611; p = 0.009), control and 3,000 ppm (Z: -2.611; p = 0.009), control and 5,000 ppm (Z: -2.611; p = 0.009) and between 1,000 and 5,000 ppm (Z: -2.611; p = 0.009) were statistically significant. Conversely, the differences between 1,000 and 3,000 ppm (Z: -1.149; p = 0.251) and between 3,000 and 5,000 ppm (Z: -1.149; p = 0.251) were not significant for the TAA/TNA ratio (Table 2).

When mean AgNOR number was taken into account, statistically significant differences were detected between control and 1,000 ppm (Z: -2.611; p = 0.009), control and 3,000 ppm (Z: -2.611; p = 0.009), control and 5,000 ppm (Z: -2.611; p = 0.009) and between 1,000 and 3,000 ppm (Z: -2.514; p = 0.012). Conversely, the differences between 1,000 and 5,000 ppm (Z: -0.419; p = 0.675) and between 3,000 and 5,000 ppm (Z: -1.776; p = 0.076) were not significant for mean AgNOR number (Table 2). In addition, statistically significant differences were detected between all groups for COHb (χ^2 : 16.995; p = 0.001) (Table 3).

To detect the relationship between COHb levels and AgNOR amounts, a polynomial regression analysis was carried out, with a statistically significant relationship being detected between COHb levels and mean AgNOR number in brain cells (p < 0.05) (Table 4, Fig. 1C). In addition, the relationship between brain cells TAA/TNA ratio and COHb levels was also statistically significant (p < 0.05) (Table 4, Fig. 1D).

Table 1. Argyrophilic nucleolar organizer regions (AgNORs) mean number, total AgNOR area/total nuclear area (TAA/TNA) ratio of each subgroup and their mean AgNOR number and TAA/TNA ratio

Groups	Mean AgNOR number $\pm$ SD (n = 50)	Mean TAA/TNA $\pm$ SD (n = 50)	Group AgNOR number average/median (range) (n = 250)	Group TAA/TNA average/ median (range) (n = 250)	χ^2	p
C1	1.56 $\pm$ 0.760	0.066 $\pm$ 0.017	1.565 $\pm$ 0.071/1.56 (0.19)	0.063 $\pm$ 0.002/0.064 (0.006)	14.339* 14.417†	0.002* 0.002†
C2	1.46 $\pm$ 0.579	0.060 $\pm$ 0.019				
C3	1.549 $\pm$ 0.541	0.064 $\pm$ 0.015				
C4	1.647 $\pm$ 0.796	0.064 $\pm$ 0.070				
C5	1.608 $\pm$ 0.532	0.063 $\pm$ 0.016				
A 1,000 ppm 1	2.647 $\pm$ 0.700	0.090 $\pm$ 0.028	2.675 $\pm$ 0.140/2.686 (0.39)	0.096 $\pm$ 0.007/0.097 (0.016)		
A 1,000 ppm 2	2.863 $\pm$ 0.663	0.104 $\pm$ 0.030				
A 1,000 ppm 3	2.706 $\pm$ 0.807	0.097 $\pm$ 0.025				
A 1,000 ppm 4	2,471 $\pm$ 0.731	0.101 $\pm$ 0.025				
A 1,000 ppm 5	2.686 $\pm$ 0.787	0.088 $\pm$ 0.024				
A 3,000 ppm 1	2.420 $\pm$ 0.785	0.095 $\pm$ 0.020	2.241 $\pm$ 0.216/2.275 (0.49)	0.107 $\pm$ 0.015/0.112 (0.034)		
A 3,000 ppm 2	2.275 $\pm$ 0.723	0.118 $\pm$ 0.057				
A 3,000 ppm 3	2.059 $\pm$ 0.580	0.122 $\pm$ 0.037				
A 3,000 ppm 4	1.98 $\pm$ 0.589	0.096 $\pm$ 0.015				
A 3,000 ppm 5	2,471 $\pm$ 0.674	0.112 $\pm$ 0.101				
A 5,000 ppm 1	2.961 $\pm$ 1.039	0.113 $\pm$ 0.049	2.678 $\pm$ 0.445/2.706 (1.16)	0.123 $\pm$ 0.016/0.116 (0.037)		
A 5,000 ppm 2	2.039 $\pm$ 0.871	0.105 $\pm$ 0.032				
A 5,000 ppm 3	3.196 $\pm$ 1.200	0.142 $\pm$ 0.057				
A 5,000 ppm 4	2.706 $\pm$ 1.064	0.138 $\pm$ 0.050				
A 5,000 ppm 5	2.490 $\pm$ 1.027	0.116 $\pm$ 0.034				

*For mean AgNOR number.

[†]For TAA/TNA.

C: control; A: acute; SD: standard deviation.

Table 2. Results of the groups double comparison for total AgNOR area/total nuclear area ratio (TAA/TNA) and argyrophilic nucleolar organizer regions (AgNORs) mean number

	Groups	C		1,000 ppm		3,000 ppm		5,000 ppm	
		Z	p	Z	p	Z	p	Z	p
TAA/TNA	C	–	–	–2.611	0.009	–2.611	0.009	–2.611	0.009
	1,000 ppm	–2.611	0.009	–	–	–1.149	0.251	–2.611	0.009
	3,000 ppm	–2.611	0.009	–1.149	0.251	–	–	–1.149	0.251
	5,000 ppm	–2.611	0.009	–2.611	0.009	–1.149	0.251	–	–
Mean AgNOR number	C	–	–	–2.611	0.009	–2.611	0.009	–2.611	0.009
	1,000 ppm	–2.611	0.009	–	–	–2.514	0.012	–0.419	0.675
	3,000 ppm	–2.611	0.009	–2.514	0.012	–	–	–1.776	0.076
	5,000 ppm	–2.611	0.009	–0.419	0.675	–1.776	0.076	–	–

C: control.

Figure 1. Comparison of argyrophilic nucleolar organizing regions (AgNOR) mean number (A) and total AgNOR area/total nuclear area (TAA/TNA) ratio (B) of the groups. Relationship between brain cells' mean AgNOR number and carboxyhemoglobin (COHb) levels (C) and between brain cells' mean TAA/TNA ratio and COHb levels (D).

Experimental demonstration of carbon monoxide toxicity in vivo

Brain hypoxia caused by carbon monoxide poisoning induces cell damage and a response is produced in the nucleus. rDNA genes are replicated from the nucleus, rRNAs are transcribed from rDNA, and NORs are translated from rRNA. NOR stained with silver in brain cells exposed different CO levels (control and 1,000, 3,000 and 5,000 ppm for brain cells) (Fig. 2).

Discussion

The appearance of inflammation, oxidative stress, oxygen radicals and apoptosis is comparable to a reperfusion injury and constitutes a mechanism of significant harm. Neurologic lesions will occur in the long term (concentration deficit, dementia, ataxias or abnormal behavior, etc.). Changes can be observed in the globus pallidus and subcortical structures. The severity of initial poisoning cannot be correlated with the development of long-term neuronal damage¹.

Oxygen concentrations can be reduced by different internal and external agents such as CO poisoning. This situation causes hypoxia and acidosis of different cells such as those of the heart, lung, muscle, brain, etc. In this condition, structural and functional damage of different tissues and cells can occur depending on the duration, time and degree of hypoxia. Therefore, early detection of the duration, time and degree of

hypoxia is of great importance not only for reducing hypoxia-induced damage, but also for the development of an efficacious treatment strategy.

Measurements of AgNOR protein amounts (especially the TAA/TNA ratio) by means of software provide more reliable results about the changes of these proteins. The AgNOR staining technique is an efficacious, reliable, inexpensive and sensitive method for evaluating ischemic brain damage. In addition, AgNORs are a good indicator for the detection of proliferation, metabolic activity, ribosomal subunits formation, protein synthesis capacity of cells, and self-renewal potential of cells damaged by ischemic events. For this reason, various studies have been carried out on different cells such as human hair root cells⁶, buccal epithelial cells in infants with Down syndrome⁷ and healthy subjects⁸, benign and malignant thyroid cells⁹⁻¹², different types of cancer cells¹³⁻¹⁵ and hypoxia caused by CO poisoning on different cells^{2,3,16-18}. According to our results, in the first study in the literature on brain exposure to CO poisoning, statistically significant differences were detected between all groups both for TAA/TNA ratio and mean AgNOR number. According to the double comparison of groups, the differences between control and 1,000 ppm, control and 3,000 ppm, control and 5,000 ppm and between 1,000 and 5,000 ppm were statistically significant for the TAA/TNA ratio. In addition, statistically significant differences were detected between control and 1,000 ppm, control and

Table 3. Comparison of carboxyhemoglobin (COHb) levels in all groups

Groups	Mean COHb (n = 50)	Group COHb average/median (range) (n = 250)	χ^2	p
C1	3	2.6 ± 0.894/3 (2)	16.995	0.001
C2	3			
C3	3			
C4	3			
C5	1			
A 1,000 ppm 1	29	29.2 ± 0.837/29 (2)		
A 1,000 ppm 2	28			
A 1,000 ppm 3	30			
A 1,000 ppm 4	30			
A 1,000 ppm 5	29			
A 3,000 ppm 1	43	42.8 ± 2.490/43 (6)		
A 3,000 ppm 2	42			
A 3,000 ppm 3	45			
A 3,000 ppm 4	45			
A 3,000 ppm 5	39			
A 5,000 ppm 1	52	49.8 ± 6.221/52 (16)		
A 5,000 ppm 2	39			
A 5,000 ppm 3	55			
A 5,000 ppm 4	51			
A 5,000 ppm 5	52			

C: control; A: acute.

Figure 2. Carbon monoxide (CO) poisoning experimental demonstration in vivo. Brain hypoxia induced by carbon monoxide poisoning causes cell damage and a response is produced in the nucleus. Ribosomal DNA (rDNA) genes are replicated from the nucleus, ribosomal RNAs (rRNAs) are transcribed from rDNA, and nucleolar organizer regions (NOR) are translated from rRNA. NORs stained with silver in brain cells exposed to different levels of CO. **A:** control; **B:** 1,000 ppm; **C:** 3,000 ppm; **D:** 5,000 ppm for brain cells (X100 magnification).

Table 4. Model summary and parameter estimates for carboxyhemoglobin (COHb) levels and argyrophilic nucleolar organizing regions (AgNOR) mean numbers and total AgNOR area/total nuclear area ratio (TAA/TNA)

Variable	Equation	Model summary					Parameter estimates			
		R ²	F	df1	df2	sig	Constant	b1	b2	b3
M-AgNOR-N and COHb	Linear	0.567	23.544	1	18	0.000	-30.679	26.982		
	Log	0.615	28.803	1	18	0.000	-17.556	60.703		
	Cubic	0.665	16.843	2	17	0.000	-144.662	134.271	-23.926	0.000
TAA/TNA and COHb	Linear	0.831	88.719	1	18	0.000	-36.059	691.112		
	Logarithmic	0.874	124.603	1	18	0.000	184.976	65.081		
	Cubic	0.885	65.595	2	17	0.000	-96.421	2019.695	-6856.583	0.000

M-AgNOR-N: mean AgNOR number.

3,000 ppm, control and 5,000 ppm and between 1,000 and 3,000 ppm for mean AgNOR number.

Previously, we reported that the level of AgNOR proteins increased depending on the duration and levels of CO exposure in the lung², myocytes^{3,16} and muscle cells^{17,18}. In the present study, the levels of AgNOR proteins also did significantly increase depending on the levels of CO exposure in brain cells. In addition, the relationships between COHb levels and AgNOR amounts (mean AgNOR numbers and TAA/TNA ratio) were statistically significant.

All living organisms must protect themselves from external and internal dangerous agents such as CO poisoning. Can AgNOR proteins be synthesized against CO poisoning for protective purposes or trigger the synthesis of other proteins that have a protective effect on the regulation of signal and gene transduction pathways in CO-poisoning conditions? Can these proteins be used as therapeutic agents to prevent the dangerous effects of CO poisoning? Hypoxia and asphyxia are a major health problem faced by humanity and can cause serious damages to the organism's life. Therefore, can the administration of these proteins to individuals at risk of hypoxic and asphyxiating birth, such as oral B12 and folate, reduce the sequelae? Can deficiencies of some organs during embryonic morphogenesis be reduced using those proteins? Further studies including large series are needed for this issue to be more clearly understood.

In conclusion, AgNORs provide information on the metabolic activity, proliferation and self-renewal potential of cells damaged by ischemic events. Furthermore, AgNOR proteins can be used for early detection of the duration, intensity and damage of CO poisoning in brain

cells. Therefore, effective treatment strategies can be developed for the prevention of hypoxic conditions.

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Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures that were followed adhered to the ethical standards of the responsible committee for experimentation on human beings and were in agreement with the World Medical Association and the Declaration of Helsinki.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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