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Evaluation of serum nitric oxide before and after local radiofrequency thermal ablation for hepatocellular carcinoma

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KEYWORDS

Nitrous oxide;
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Abstract *Background:* HCC is one of the leading causes of world wide cancer mortality due to late diagnosis. Chronic hepatitis C virus is one of the main risk factors for the development of Hepatocellular carcinoma (HCC), which is a multi-step process involving different genetic alterations that lead to malignant transformation of hepatocytes. Genetic and molecular abnormalities associated with viral infection or due to inflammatory conditions represent an early step in hepatocarcinogenesis. HCC is a hypervascular solid cancer. Tumor growth depends on angiogenesis, and the “angiogenic switch” of preexisting vessels is required to allow tumor progression, growth, and propagation to supply nutrients and oxygen. Inducible nitric oxide synthase (iNOS) also plays an important role in angiogenesis, regulating several biological processes crucial for tumor growth.

Objectives: Evaluation of serum nitric oxide before and after local radiofrequency thermal ablation for hepatocellular carcinoma.

Subjects: Twenty patients with proven hepatocellular carcinoma and 15 healthy patients as controls were enrolled in the study.

Abbreviations: NO, nitrous oxide; HCC, hepatocellular carcinoma; RF, radiofrequency

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Methods: History taking, clinical examination, laboratory testing (AIT, AST, Bil γ GT, ALP, Albumin, AFP, NO), ultrasound and Spiral CT. Evaluation was done initially and repeated after 2 weeks of tumor ablation by local radiofrequency thermal ablation.

Results: Median of Serum Nitric oxide was statistically significantly higher among HCC patients before radiofrequency thermal ablation (1200 μ mol/l) compared to controls (22 μ mol/l) where $p < 0.001$, also the median of NO was statistically significantly declined after radiofrequency thermal ablation compared to before (160, 1200 μ mol/l) respectively where $p < 0.001$.

Conclusion: The data suggest that there is an elevation in serum nitric oxide in HCC patients and that is locally produced from the tumor and hence its level significantly drops after local radiofrequency thermal ablation.

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1. Introduction

Primary liver cancer PLC is the fifth most common cancer worldwide with approximately 600,000 deaths annually. Hepatocellular carcinoma (HCC) accounts for approximately 85–90% of all PLC, out of which 80% of HCC cases occur in either sub-Saharan Africa or in eastern Asia.^{1,2} Risk factors that lead to the multistep development of HCC are well known and it is established that approximately 80% of HCC cases develop in individuals suffering from chronic hepatitis B or C viral infection HBV or HCV, cirrhosis, and also those with a high exposure to aflatoxin-B1 as well as those with a high intake of alcohol.^{3–5}

In a setting of chronic inflammation, cytokines and reactive oxygen and nitrogen species produced by inflammatory cells have been shown to mediate liver damage and induce the liver's regenerative response. This predisposes the proliferating cells to a variety of genetic changes at the genomic and transcriptional levels.^{6–10}

The main sources of reactive species in cells are mitochondria, cytochrome P450 and peroxisome. Under physiological conditions, there is a constant endogenous production of reactive oxygen ROS and nitrogen species and RNS that interact as “signaling” molecules for metabolism, cell cycle and intercellular transduction pathways.¹¹

To control the balance between the production and removal of ROS, as hydroxyl and superoxide radicals, and RNS, as nitric oxide, peroxynitrite and S-nitrosothiols, there are a series of protective molecules and systems globally defined as “antioxidant defences”. Oxidative stress occurs when the generation of free radicals and active intermediates exceeds the system's ability to neutralize and eliminate them. In these conditions, ROS and RNS affect the intracellular and intercellular homeostasis, leading to DNA and protein oxidation, cell membrane damage, gene mutation, gene damage implicated in cell growth, cell cycle, apoptosis, disruption of DNA repair pathways as well as possible cell death. These changes render the cells more susceptible to spontaneous or mutagen induced alterations.¹²

Therefore, free radical production and oxidative injury, constitute the first step of a cascade of epigenetic aberrant DNA methylation, genomic point mutations and post-genomic protein oxidation and cytokine synthesis, events that lead to HCC.^{13–15}

Reactive species also play an important role in fibrogenesis throughout the increasing of the platelet derived growth factor or the secretion of profibrotic cytokines, such as TGF- β . Thus,

oxidative stress plays an evident role in the progression of liver fibrosis and cirrhosis.^{15,16}

NO, a small potent lipophilic gas with divergent biological activities, seems to play an important role in modulating tissue injury and carcinogenesis. Three distinct forms of nitric oxide synthase (NOS) catalyze the formation of NO. Endothelial NOS and neuronal NOS are constitutively expressed in different tissues, whereas inducible nitric oxide synthase (iNOS) is related to a high-output pathway for NO production which contributes to tumor cell angiogenesis as well as the invasion and metastases of HCC.^{17,18}

Neoplastic tissue requires a supply of oxygen and nutrients to continue its growth and meet its metabolic demands. Thus, it is necessary for a tumor to orchestrate the formation of a functioning system of blood vessels, which allows the delivery of metabolites including growth factors and cells as immunological cells and other cellular precursors to the tumor environment.

Newly formed vessels have abnormal architecture and tend to facilitate the spread of tumor cells. If disseminated tumor cells become located in other tissues, the whole process of tumorigenesis may reoccur with the generation of secondary tumors.^{19,20}

The formation of new functional blood vessels occurs in several phases including endothelial cell budding which is facilitated by vasodilatation, loosening of interendothelial contacts and leakage from pre-existing vessels. These phenomena allow extravasation of plasma proteins that together with the extracellular matrix components facilitate the laying down of a provisional scaffold for migrating endothelial cells.^{21,22}

HCV infection causes elevated iNOS transcription which might be responsible for carcinogenesis in the cirrhotic liver and these effects depend on NO concentrations.²³

Inflammation induces the expression of iNOS, which results in an increased production of nitric oxide and nitrosoglutathione (GSNO), which is degraded by a reductase, GSNOR. GSNO is in equilibrium with nitrosylated proteins, among them the DNA repair enzyme AGT, which is then degraded by the proteasome. Nitroso compounds lead to the formation of *O*⁶-alkylguanines, which, if not repaired by AGT, will cause mutagenesis and favor the tumorigenic processes and hepatocellular carcinoma²⁴ (fig. 1).

Radio frequency ablation (RFA) therapy is one of the best curative treatment options for malignant liver tumors, and can be an alternative to resection. RFA can be performed safely using percutaneous, laparoscopic or open surgical techniques as RFA has markedly changed the treatment strategy for small

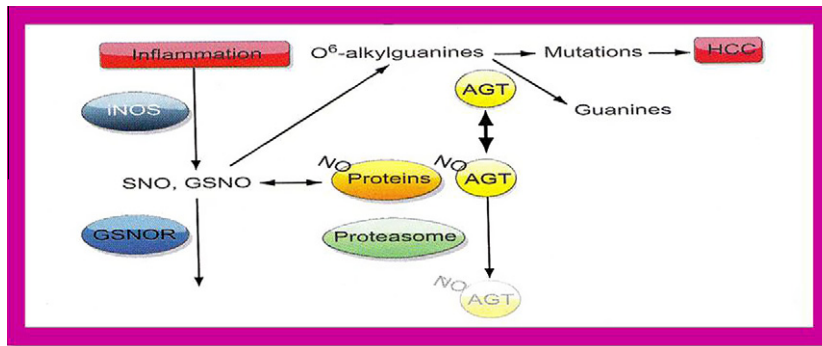


Figure 1 Inflammation induces the expression of iNOS.

HCCs. Currently, RFA has gained popularity based on the ease of use, safety, reasonable cost and applicability. Localized application of thermal energy induces tumor cell destruction. When tumor cells are heated above 50 °C, intracellular proteins are denatured and cell membranes are destroyed through the dissolution and melting of lipid bilayers.²⁵

RFA is a localized thermal treatment technique designed to produce tumor destruction by heating tumor tissue to temperatures that exceed 60 °C. The alternating current of RF waves passing down from the uninsulated electrode tip into the surrounding tissues generates changes in the direction of ions and creates ionic agitation and frictional heating, this tissue heating then drives extracellular and intracellular water out of the tissue resulting in tissue destruction by coagulative necrosis.^{26,27}

2. Aim of work

Evaluation of the serum level of nitric oxide (NO) in patients with HCC; before and 2 weeks after local radiofrequency thermal ablation is done proving that HCC is the main source of NO in the case of HCC complicating cirrhosis.

3. Subjects and methods

The present study included 20 patients diagnosed as HCC on top of liver cirrhosis who were presented to the Hepatobiliary Unit, Alexandria Main University Hospital, and 15 matched normal subjects as a control to obtain the normal range of biochemical parameters. Written consent was taken from all participants included in the study before starting the research.

All patients included in the study were subjected to the following:

- Full history taking, clinical examination to assess liver, spleen, ascites, lower limb edema together with complete liver profile ALT,²⁸ AST,²⁹ serum bilirubin,³⁰ γ Gt,³¹ ALP,³² serum albumin,³³ prothrombin activity,³⁴ serum alpha fetoprotein,³⁵ HBsAg,³⁶ HCVAb³⁷ and Child-Pugh score.³⁸
- Examination of the liver using B-mode standard ultrasonography scanning to assess liver condition as regards: cirrhosis and presence of focal lesion(s). Also, the number; site as well as size of the lesion(s).³⁹ The information obtained was confirmed by performing triphasic CT of the abdomen.⁴⁰

- Different types of RF electrodes are currently available, the one used in the present study belongs to the RITA medical system. The needle electrode of RITA consists of a 14-gauge insulated outer needle that houses nine retractable curved electrodes of various lengths. When the electrode is extended, the device assumes the approximate configuration of a Christmas tree. The alternating electric current generator comes in a 250-W model at 460 kHz (Model 1500-X RF Generator, RITA Medical System).⁴¹

All procedures were performed percutaneously using sonographic guidance. For lesions in the right lobe, an intercostal approach was used, whereas lesions in the left lobe were treated with an epigastric approach.

Serum NO level⁴² was measured in HCC patients who were candidates to RFA therapy according to BCLC staging classification, before and 2 weeks after complete ablation.

Statistical analyses were performed using SPSS version 13 for windows program. Chicago, SPSS incorporation 2000.⁴³

4. Results

Table 1 showed the clinical data of patients and radiological characteristics before RF. Liver was shrunken in 6 (30%) and enlarged in 14 (70%) patients. Six (30%) patients had ascites and five (25%) had jaundice, none of the patients had encephalopathy. The tumor size ranged from 2 to 4.5 cm with a median value of 3 cm. Table 2 shows: the median values of the laboratory results of the present patients for GPT, GOT, γ GT, Bilirubin total, direct, albumin and alkaline phosphatase before RF were significantly higher 47U/L,65U/L,40U/L,1.6 mg/dl,1.2 mg/dl, 3.30 g/dl,88U/L than after RF which were 27.5U/L,35U/L,15U/L,1.1 mg/dl,0.15 mg/dl, 5.15 g/dl,35.5U/L respectively, where $p < 0.001$.

Table 3 & Fig. 2; shows a significant difference between NO in cases gp before RF which showed a median value of 1200 μ mol/l than the control gp median value 22 μ mol/l where $p < 0.001$, also there was a significant difference between NO after RF which had a median value of 160 μ mol/l than that of the control median value 22 μ mol/l where $p < 0.001$.

AFP median value before RF was significantly higher 95 ng/l than after RF which was 10 ng/l where $p < 0.001$, this was evident in Fig. 3.

Table 4; Spearman's correlation showed no significant correlation between NO and AFP before and after RF where $p = 0.053$ and $p = 0.449$, respectively. Table 5 shows that no

significant correlation exists between AFP and NO with tumor size where $p = 0.867$ and $p = 0.717$ respectively. Table 6 represented the clinical classification according to Child-Pugh score where 14 (70%) patients were of class A and 6 (30%) patients were of class B, evidently no significant correlation exists between Child-Pugh, NO and AFP before RF.

5. Discussion

Mammalian cells have the ability to synthesize the free radical nitric oxide (NO) which stimulated an extraordinary impetus for scientific research in all the fields of biology and medicine. Since its early description as an endothelial-derived relaxing factor, NO has emerged as a fundamental signaling device regulating virtually every critical cellular function, as well as a potent mediator of cellular damage in a wide range of conditions.⁴⁴

Recent evidence indicates that most of the cytotoxicity attributed to NO is rather due to peroxynitrite, produced from the diffusion-controlled reaction between NO and another free radical, the superoxide anion. Peroxynitrite interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms.⁴⁵

These reactions trigger cellular responses ranging from subtle modulations of cell signaling to overwhelming oxidative injury, committing cells to necrosis or apoptosis, representing a crucial pathogenic mechanism in cancer.^{46,47}

In the present study it is clearly evident that NO median level was significantly higher in the studied patient group before and after RF than matched normal control group 1200, 160 $\mu\text{mol/l}$, 22 $\mu\text{mol/l}$, respectively where $p < 0.001$. (Tables 1 and 3).

Parasole et al.⁴⁸ stated that AFP appeared in many parenchymatous liver diseases such as acute viral hepatitis and chronic

Table 1 Clinical data of patients before RF.

Clinical & radiologic parameters	No.	%
<i>Liver</i>		
Shrunken	6	30.0
Enlarged	14	70.0
<i>Spleen</i>		
Not enlarged	3	15.0
Enlarged	17	85.0
<i>Ascites</i>		
-ve	14	70.0
+ve	6	30.0
<i>Jaundice</i>		
-ve	15	75.0
+ve	5	25.0
<i>Segmental</i>		
V	4	20.0
VI	4	20.0
VII	9	45.0
VIII	3	15.0
<i>Tumor size(cm)</i>		
Range	2.0–4.50	
Mean \pm SD	3.05 \pm 0.74	
Median	3.0	

Table 2 Laboratory data of patients before and after RF.

	Before (RF)	After (RF)	<i>p</i> value
<i>GPT (U/L)</i>			
Range	15.0–91.0	15.0–52.0	< 0.001*
Mean \pm SD	46.25 \pm 17.94	28.10 \pm 9.98	
Median (IQR)	47.0 (26.75)	27.50 (14.25)	
<i>GOT (U/L)</i>			
Range	27.0–88.0	25.0–78.0	< 0.001*
Mean \pm SD	65.35 \pm 15.38	39.45 \pm 14.07	
Median (IQR)	65.0 (20.0)	35.0 (17.25)	
γ <i>GT (U/L)</i>			
Range	14.0–153.0	10.0–124.0	< 0.001*
Mean \pm SD	64.50 \pm 48.53	24.50 \pm 24.52	
Median (IQR)	40.0 (87.25)	15.0 (12.0)	
<i>Bil (total) (mg/dl)</i>			
Range	1.20–4.10	0.50–2.0	< 0.001*
Mean \pm SD	1.93 \pm 0.79	1.12 \pm 0.43	
Median (IQR)	1.60 (0.93)	1.10 (0.18)	
<i>Bil (Direct) (mg/dl)</i>			
Range	0.60–2.90	0.10–1.70	< 0.001*
Mean \pm SD	1.28 \pm 0.55	0.44 \pm 0.52	
Median (IQR)	1.20 (0.85)	0.15 (0.45)	
<i>CBC: white count</i>			
Range	2100.0–4800.0	3000.0–5250.0	0.012*
Mean \pm SD	3110.0 \pm 698.80	352250 \pm 642.46	
Median (IQR)	3000.0 (600.0)	3500.0 (550.0)	
<i>Platelets (*10)³</i>			
Range	70.0–230.0	150.0–350.0	0.001*
Mean \pm SD	14.68 \pm 59.76	213.65 \pm 51.93	
Median (IQR)	15.20 (125.0)	220.0 (925.0)	
<i>Albumin (gm/dl)</i>			
Range	1.50–5.10	2.80–5.80	< 0.001*
Mean \pm SD	3.25 \pm 1.0	4.68 \pm 0.96	
Median (IQR)	3.30 (0.98)	5.15 (1.85)	
<i>ALK (U/L)</i>			
Range	60.0–335.0	19.0–162.0	< 0.001*
Mean \pm SD	108.20 \pm 72.17	46.65 \pm 30.62	
Median (IQR)	88.0 (53.50)	35.50 (27.0)	
<i>Proth time (s)</i>			
Range	12.80–19.60	12.80–13.80	0.016*
Mean \pm SD	15.36 \pm 2.52	13.57 \pm 0.23	
Median (IQR)	13.80 (4.35)	13.50 (0.30)	
<i>Proth activity (%)</i>			
Range	40.0–72.0	70.0–72.0	0.003*
Mean \pm SD	59.65 \pm 14.75	71.30 \pm 0.57	
Median (IQR)	70.0 (31.25)	71.0 (1.0)	
<i>AFP (ng/ml)</i>			
Range	4.0–200.0	2.0–68.0	< 0.001*
Mean \pm SD	92.90 \pm 57.09	20.45 \pm 20.33	
Median (IQR)	95.0 (99.75)	10.0 (32.50)	
<i>NO ($\mu\text{mol/L}$)</i>			
Range	660.0–1700.0	110.0–450.0	< 0.001*
Mean \pm SD	1180.50 \pm 263.31	200.0 \pm 93.41	
Median (IQR)	1200.0 (375.0)	160.0 (115.0)	

p: *p* value for Wilcoxon signed ranks test.

* Statistically significant at $p \leq 0.05$.

hepatitis but higher levels were found most frequently in HCC. This was in agreement with our present study where AFP median

Table 3 Comparisons between cases and control according to the nitric oxide before and after RF.

Variables	Cases	Control	Mann–Whitney test values (<i>P</i>)
<i>Nitric oxide (before)</i>			
Min.–max.	660–1700	20–25	5.014* (<0.001)
Mean ± SD	1180 ± 263.3	22.2 ± 2	
Median (IQR)	1200 (375)	22 (5)	
<i>Nitric oxide (after)</i>			
Min.–max.	110–450	20–25	5.019* (<0.001)
Mean ± SD	200 ± 93.4	22.2 ± 2	
Median (IQR)	160 (115)	22 (5)	

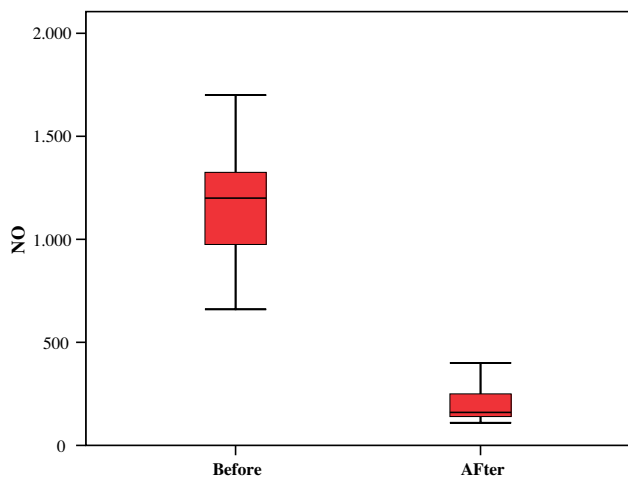


Figure 2 Box plot presentation for NO before and after RF.

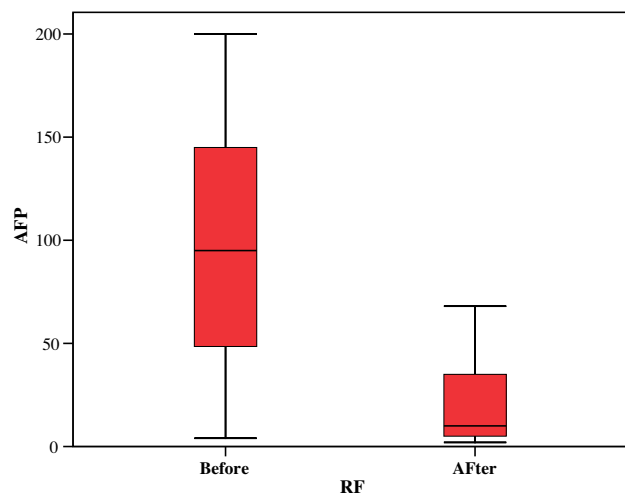


Figure 3 Box plot presentation for AFP before and after RF.

value was significantly higher in the studied HCC patient group before RF 95 ng/ml than the matched control group median value 22 ng/ml where $p < 0.001$ and after the RF median value which was 10 ng/ml where $p < 0.001$. (Table 2).

Relationship between chronic inflammation and tumorigenesis has been long suspected. It is well known that malignant tissues are infiltrated by leukocytes, which locally secrete cytokines, chemokines, matrix-degrading enzymes,

Table 4 Correlation between NO and AFP before and after radiofrequency (RF).

	NO			
	Before RF		After RF	
	Rho	<i>p</i>	Rho	<i>p</i>
AFP	0.439	0.053	0.179	0.449

Rho (ρ): Spearman coefficient.

Table 5 Correlation between tumor size, NO and AFP before (RF).

	Tumor size	
	Rho	<i>p</i>
AFP (ng/ml) before RF	0.040	0.867
NO (μ mol/L) before RF	0.086	0.717

Rho (ρ): Spearman coefficient.

growth factors, free radicals, and oxidants. This creates a microenvironment that may enhance cell proliferation, survival, migration, as well as angiogenesis, thereby promoting tumor development.⁴⁹

A particularly important role of increased NO generation in this microenvironment is now well recognized as an essential step initiating neoplastic transformation.⁵⁰ Importantly, not only immune cells infiltrating the tumor, but the tumor cells themselves, are able to produce large amounts of NO due to induced expression of iNOS, which may prevail in rapidly growing tumors.⁵¹ Evidence for a role of NO overproduction as a mechanism initiating and promoting tumorigenesis is seen,⁵² also Qiang et al.⁵³ reported that the double edged sword of NO in tumor biology clearly depends on cell type, NO concentration, oxidative stress and tumor milieu.

This was documented in our study by the significant elevated median level of NO in the HCC patient group before RF 1200 μ mol/l than the control median value 22 μ mol/l and was significantly reduced after ablation of the tumor cells by RF which was 160 μ mol/l indicating that the tumor cells are the primary site for the excess NO production.

Tumor-promoting influence of NO has been identified, it can stimulate tumor angiogenesis, by inducing angiogenic and lymphangiogenic factor expression, most significantly vascular endothelial growth factor VEGF^{54–56} and by stimulating blood vessel maturation via the recruitment of perivascular cells pericytes,^{57,58} also NO has been associated with

Table 6 Relation between Child–Pugh score with AFP and NO before RF.

	Child–Pugh score		<i>p</i> value
	Class (A)	Class (B)	
AFP (ng/ml) before RF			
Range	4.0–170.0	5.0–200.0	0.200
Mean ± SD	82.57 ± 50.07	117.0 ± 69.77	
Median (IQR)	80.0 (78.50)	135.0 (114.0)	
NO (µmol/L) before RF			
Range	660.0–1700.0	860.0–1500.0	0.934
Mean ± SD	1180.0 ± 280.38	1181.67 ± 243.02	
Median (IQR)	1200.0 (337.50)	1220.0 (467.50)	

p: *p* value for Mann Whitney test.

enhanced migration and invasion of tumor cells through mechanisms depending on guanylyl cyclase and MAPK signaling.^{59,60} These influences of NO depend on the duration and level of NO exposure, the type of iNOS-expressing cells and cellular sensitivity to nitric oxide cytotoxic activity.⁵⁰

Masahide et al.⁶¹ reported that the overproduction of NO in malignant tissues by iNOS inhibits the immune defence mechanism and increases tumor blood, correlating with carcinogenesis in cirrhotic liver but does not play a role in tumor progression in HCC. This was in accordance with our present study where there was no significant correlation between NO before RF and the tumor size.

6. Conclusion

Overproduction of NO may represent an essential link between inflammation and carcinogenesis. Available evidence indicates that NO plays a crucial role in tumorigenesis. Significant reduction of NO level is a marker for ablation of HCC by RF.

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