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Full Length Research Paper

Comparative study on the protective effect of carnosine and carnitine against pro-inflammatory/pro-oxidant effects of clindamycin and propionic acid administrations to hamsters

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This study aimed to compare the neuroprotective potency of carnosine and carnitine supplements against the proinflammatory and prooxidant effects of orally administrated propionic acid and clindamycin induced changes in microflora of hamsters. Nine experimental groups, each consisting of six young male golden Syrian hamsters were investigated: control group (received only phosphate buffered saline), oral buffered PA-treated group (received a neurotoxic dose of 250 mg/kg PA for three days), oral clindamycin-treated group (received a single dose of 30 mg clindamycin/kg), oral carnosine-treated group received a daily dose of 10 mg/kg/day for one week, oral carnitine-treated group was given a daily oral dose of 50 mg/kg/day for one week, and four protected groups received the same doses of carnosine or carnitine for one week followed by PA for 3 days or a single dose of clindamycin. Interferonγ (INF-γ), heat shock protein (HSP70), malondialdehyde (MD), and glutathione peroxidase (GPX) were measured in the cortexes and medullas at the end of the experiment. This study confirmed the pro-inflammatory and pro-oxidant effects of orally administered PA and overgrowth of pathogenic bacteria in hamsters. While PA induced a significant increase of IFN-γ, HSP70, and MD with a significant decrease of GPX activity in brain cortex and medulla, clindamycin brought a marked increase only in MD and HSP70 with a reduction of GPX activity. Receiver operating characteristics (ROC) analysis showed satisfactory specificity and sensitivity which could help to suggest that the measured parameters could be used as brain injury biomarkers. Although bacterial overgrowth induced by clindamycin was effective in inducing signs of neuronal toxicity in cortex and medulla, its effect were not comparable to those induced by the oral administration of PA. The findings suggest that the overgrowth of specific bacteria does not manifest significant neurologic harms until there is toxic exposure to a dietary metabolite.

Key words: Autism, propionic acid, clindamycin, oxidative stress, pro-inflammation, bacterial overgrowth.

INTRODUCTION

The gut-brain axis (GBA) is a bidirectional neurohumoral communication system that integrates brain and gastrointestinal (GI) functions. Several links have been established between GBA and the pathophysiology of

functional GI disorders, and evidence is emerging for the role of blood brain axis (BBA) in the pathogenesis of gut inflammatory disorders, such as inflammatory bowel diseases.

There are several mechanisms by which stress can alter the bacterial composition of the GI tract, including alterations in epithelial cell function and mucus secretion as well as changes in GI motility (Varghese et al., 2006; Groot et al., 2000). The release of norepinephrine into the GI tract during stress was previously described to preferentially induce the growth of specific strains of bacteria and to enhance their adhesion abilities to the mucosa (Freestone et al., 2002). Several studies have also shown that the brain responds to the introduction of noninvasive pathogenic bacteria into the cecum, that brain stem nuclei are rapidly activated, and that there is expression of anxiety-like behavior in mice (Goehler et al., 2007). The relationship between the microbiota and autistic behavior remains, however, in the main speculative and inconclusive.

A common neural developmental disorder, autism affects brain functions and manifests itself in several cognitive, affective, and behavioral symptoms, including delays in language skills, deficiencies in social interaction, appearance of aberrant repetitive movements, hyperactivity, disturbances in sensory processing, restricted interests, and, occasionally, incidences of self injury (Zwaigenbaum et al., 2005). Many studies show increased levels of pro-inflammatory cytokines in brain, cerebrospinal fluid (CSF), and blood of autistic children (El-Ansary et al., 2011; Ashwood et al., 2011). Inflammatory cytokines including tumor necrosis factor (TNF-α), interferon (IFN-γ), interleukin (IL)-1β and IL-12 are elevated in the blood mononuclear cells, serum and plasma of autistic subjects.

There is increasing evidence that autistic patients present excessive reactive oxygen species (ROS) production and reduced methylation capacity. Brain tissues from autistic cases showed higher levels of oxidative stress biomarkers than healthy controls in postmortem analysis (Chauhan et al., 2011; Sajdel-Sulkowska et al., 2011). Moreover, mitochondrial abnormalities, a potential source of elevated oxidative stress were reported in autistic case studies and have been recently reevaluated through meta-analysis (Rossignol and Frye; 2012).

Interestingly, a number of behavioral and brain abnormalities that are similar to those observed in autistic patients have recently been reported in rats following the administration of intraventricular infusions of propionic acid (PA), presumably via the alteration of brain fatty acid metabolism (MacFabe et al., 2007; Shultz et al., 2008; Shultz et al., 2009). In fact, relatively large amounts of PA have been used to induce the symptoms (4 µl of 0.26 M solution).

The composition of the intestinal microbiota is extremely relevant in neurogastroenterology, which deals with the interactions of the central nervous system and the gut (gut–brain axis). Several neuropathological diseases are thought to be associated with the gut microbiota. Autism is a disorder of neural development with impaired social behavior and often involves GI symptoms. Previous studies that examined the faecal microbial profiles of autistic children, indicated 10-fold higher numbers of Clostridium spp as PA producers, compared with healthy subjects (Sekirov et al., 2010). Many species of Clostridium are known to produce neurotoxins, which could contribute to the autism spectrum. Additionally, autistic children could accumulate PA through diet as it is one of the most common food preservatives (Subils et al., 2012).

It remains however, to be ascertained whether the concentrations produced by the microbiota could lead to similar effects as those induced directly by PA. In fact, there is evidence in the literature that high levels of PA can induce oxidative stress in various brain regions (MacFabe et al., 2007). Several brain areas (neocortex, hippocampus, thalamus, and striatum) of rats treated with PA (intraventricular) have, for instance, been reported to show increased levels of lipid and protein oxidation accompanied by decreased levels of total GSH in the cortex. Catalase activity was also reported to decrease in most brain regions, suggesting a reduced antioxidant enzymatic activity (MacFabe et al., 2008). The results suggest that early postnatal PA administration to rats altered normal development and induced long-term behavioral deficiencies.

Likewise, a recent study by El-Ansary et al. (2011) on rat pups proved that orally administered neurotoxic doses of PA (250 mg/kg/day) for three days induced highly significant oxidative, inflammatory, and pro-apoptotic effects that could be related to the autistic features reported by Macfabe et al. (2008) and Shultz et al. (2008) in intraventricularly PA-induced rats.

Considering the serious concerns expressed over the alarming rates with which autism spectrum disorders are increasing worldwide and in light of the promising new opportunities that PA administration might bring with regards to the identification of aspects of neurotoxicity and understanding of the etiological factors of autism, the present study was undertaken to further understand the role played by altered microbiota with regards to the neurotoxicity of the brain.

Clindamycin was used to induce the overgrowth of pathogenic bacteria among which are short chain fatty acids (SCFAs) producers so as to explore whether the pro-inflammatory/pro-oxidant effects are comparable to those produced by orally administered PA, as a metabolite of enteric bacteria, and whether both treatments

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could induce biochemical features of autism.

MATERIALS AND METHODS

Chemicals

PA, carnosine, and carnitine, all of analytical grade, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Clindamycin was purchased from Pharmacia Co. Ltd (Peapack, NJ, USA).

Animals

A total of 54 young male golden Syrian hamsters weighing about 80-100 g (8 weeks of age) were used in the present study. The animals were randomly assigned to nine groups, each consisting of six hamsters. The control group consisted of normal hamsters that were given only phosphate buffered saline. The oral buffered PA-treated group was given a nuerotoxic dose of PA (250 mg/Kg body weight/day) for 3 days (El-Ansary and Al-Daihan, 2011). The clindamycin-treated group was given a single orogastric dose of clindamycin (30 mg/kg body weight) on day 0 of the experiment. The carnosine-treated group was given a daily oral dose of carnosine (10 mg/kg body weight/day) for one week. The Carnitine-treated group was given daily oral dose of carnitine (50 mg/kg body weight/day) for one week. The remaining four groups consisted of protected groups of hamsters that were given the same doses of carnosine or carnitine for one week followed by PA for 3 days or a single dose clindamycin as described above.

All groups were kept at controlled temperature (21 ± 1°C) with *ad libitum* access to food and water. Quantitative stool cultures were performed both aerobically and anaerobically. All the procedures used in this study were preapproved by the ethics committee of the Faculty of King Saud University, and all experiments were performed in accordance with the national guidelines for animal care and use.

Brain tissue preparation

At the end of experiment, the hamsters were anesthetized using carbon dioxide and decapitated. Their brains were carefully removed from the skull, and the cortex and medulla were extracted. The brain tissues taken from the nine groups of hamsters were homogenized in 10 times w/v bidistilled water and kept at -80°C until further use.

Biochemical analyses

*Assay of Interferon-γ (*INF-γ)

INF-γ was measured using an enzyme-linked immunosorbent assay (ELISA) kit, a product of Thermo Scientific, USA, according to the manufacturers' instructions. This assay employs a quantitative sandwich enzyme immunoassay technique that measures INF-γ in less than 5 h. A polyclonal antibody specific for human IFN*γ* was pre-coated onto a 96-well microplate with removable strips. IFN*γ* in standards and samples was sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for IFN*γ*, which was recognized by a streptavidin-peroxidase conjugate. All unbound material was then washed away three times and a peroxidase enzyme substrate was added. The color development was stopped by adding sulfuric acid, and the intensity of the color was measured at 550 nm and subtracted from absorbance at 450 nm. The minimum detectable level of rat IFNγ detected by this product was at 2 pg/ml.

Assay of glutathione peroxidase (GPX)

In this assay, cumene hydroperoxide was used as the peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and reduced β−nicotinamide adenine dinucleotide phosphate (NADPH) were included in the reaction mixture. The formation of GSSG catalyzed by GPX was coupled to the recycling of GSSG back to glutathione (GSH) using GSSG-R. NADPH was oxidized to $NADP^+$. The change in absorbance at 340 nm due to NADPH oxidation was monitored and was indicative of GPX activity.

Measurement of lipid peroxidation

The extent of lipid oxidation could be determined by measuring the levels of lipid peroxidation products, mainly malondialdehyde (MD), as thiobarbituric acid reactive substances using the method of Ruiz-Larrea et al. (1994).). According to this method, the samples were heated with TBA at low pH, and a pink chromogen was measured by its absorbance at 532 nm. The concentration of lipid peroxides was calculated as µmoles/ ml using the extinction coefficient of MD.

Assay of heat shock protein (HSP70)

HSP70 was measured in homogenates of brain cortex and medulla using an ELISA kit, a product of Uscn Life Science Inc. (Wuhan, China), according to the manufacturers' instructions. The microtiter plate provided in this kit was pre-coated with an antibody specific to HSP70. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for HSP70. After that, avidin conjugated to Horseradish Peroxidase was added to each microplate well and incubated. A TMB substrate solution was then added to each well. Only those wells that contained HSP70, biotin-conjugated antibody, and enzyme-conjugated avidin would exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of HSP70 in the samples was then determined by comparing the O.D. of the samples to the standard curve. The minimum detectable level of rat HSP70 detected was at 0.045 ng/ml.

Measurement of bacterial overgrowth

Sample collection

The caecal contents of the hamsters were collected in sterile tubes and immediately stored at -20°C. The frozen tubes were then analyzed. The process of bacterial cultivation involves the use of optimal artificial media and incubation conditions to isolate and identify the bacterial etiologies of an infection as rapidly and accurately as possible.

Bacterial / yeast *culture techniques*

Bacterial counts in feces were obtained by the methods previously described by Itoh et al. (1983). The faecal specimens were diluted 1: 10 (w/v) in pre-reduced phosphate-buffered saline (PBS) (0.1 M, pH 7 . 0; Oxoid). The faecal suspensions were thoroughly mixed by vortex mixer to produce an even suspension. For the quantification of C. *difficile,* a fecal suspension was made in pre-reduced PBS under anaerobic conditions and transferred into an anaerobic chamber (85% N_2, 10% H_2, 5% CO2) (20). Serial tenfold dilutions were made in buffered solution A (KH_2P0_4, 4 g; Na_2HP0_4, 6 g; Tween 80, 1 g; agar, 1 g; L-cysteine, 0.5 g; in 1000 ml water). Aliquots (0·1 ml of diluted stools) were spread onto modified

cefoxitin cycloserine fructose agar (CCFA) plates and incubated at 37°C for 3 days in the anaerobic chamber.

The identification of *C. difficile* was performed on the basis of colony and cell morphology using Gram staining and biochemical characteristics. For the other aerobic bacteria, portions of 0.1 ml of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were plated with a glass spreader onto the following media: 5% Sheep Blood Agar plate (BAP), MacConkey Agar (MCA), Muller Hinton Agar (MHA), and Sabouroud Dextrose agar, yeast media (SDA). The inoculated plates were incubated at 37°C overnight in aerobic conditions.

Quantification

The data from the culture-based methods were quantified based on a ++++ scale defined as follows: 0 = no growth, < 10^3 colony forming units/gram of feces; + = rare, < 10^3 colony forming units/gram of feces; ++ = few, 10^3 - 10^4 colony forming units/gram of feces; +++ = moderate, 10^5 - 10^6 colony forming units/gram of feces; ++++ = heavy, > 10^6 colony forming units/gram of feces.

Colony-forming unit (CFU) is a measure of viable bacterial or fungal numbers. Unlike direct microscopic counts where all cells, dead and living, are counted, CFU measures viable cells. For each dilution, the number of colony forming units on the plates was counted. Numbers between 30 and 300 were used to estimate the cultural count.

Identification

Every well-isolated colony, regardless of appearance, was picked in succession (top of the plate). The colonies that were so close together that they could not be picked separately or that were under an obvious area of surface spreading were not picked. If more than one colony type or more than one morpho type was observed, an attempt was made to isolate and characterize each. Each isolate was characterized according to procedures previously described elsewhere (Holdeman and Moore, 1973). The colonies were spread on the slide. Smears were heat-fixed, gently Gram stained, and then examined under a microscope using oil immersion lens.

Statistical analysis

The data were analyzed using the statistical package for the social sciences (SPSS, Chicago, IL, USA). The results were expressed as mean ± S.D. All statistical comparisons between the control and PA-treated hamsters groups were performed using the One-way ANOVA test complemented with the Dunnett test for multiple comparisons. Significance was assigned at the level of $P < 0.05$. Receiver operating characteristics curve (ROC) analysis was performed. Area under the curve (AUC), specificity, and sensitivity are demonstrated in Figure 3.

RESULTS AND DISCUSSION

One of the strategies commonly used for assessing the effect of intestinal microbiota on the functioning of the host is to perturb the commensal bacteria using oral antibiotics (Finegold, 2011). Table 1 illustrates the bacterial growth rates in the control and clindamycin-treated groups. Quantitative stool cultures were performed on groups of hamsters receiving clindamycin and untreated control. The results are summarized in Table 1. It could be easily noted that clindamycin induced the overgrowth of several aerobic and anaerobic pathogenic bacteria. In this experiment, the most striking changes in feces of clindamycin-treated hamsters, as compared with untreated controls, were a marked increase in total clostridia species, *Klebsiella pneumoniae* and group *streptococci*. The clindamycin-treated group was, therefore, taken to represent bacterial overgrowth group.

IFNγ is a cytokine that is produced primarily by activated CD^{4+} or CD^{8+} T cells and natural killer cells, and is recognized as a major mediator of innate as well as adaptive immunity (Billiau, 1996). Among the biological activities of IFNγ, the activation of macrophages is of special importance. Accordingly, IFN-γ up-regulates a variety of pro-inflammatory parameters, such as interleukin (IL)-12 (Yoshida et al., 1994), IL-15 (Doherty et al., 1996), tumor necrosis factor α (TNF-α) (Hayes et al., 1995), interferon-inducible protein-10 (Luster and Ravetch, 1987), inducible nitric oxide synthase (iNOS) (Xie et al., 1993), and caspase- 1 (Tamura et al., 1996). Moreover, current research suggests that, under certain conditions, IFNγ may also be able to enhance the activation of the pro-inflammatory transcription factor nuclear factor κB (NF-κB) (Cheshire and Baldwin, 1997). In accordance with those functional characteristics, the bioactivity of IFN γ has been identified as a prerequisite in several models of inflammatory and autoimmune diseases, such as multiple sclerosis (MS) and autism.

Table 2 and Figure 1a show that a significantly higher level of IFN-γ was recorded in both the cortex and medulla of PA-treated hamsters. This could be attributed to the fact that PA-treatment may modulate immune function through stimulating the release of pro-inflammatory cytokines, such as IFNγ (Cavaglieri et al., 2003). This modulation of the immune system may also occur via the direct activation of G-protein coupled receptors specific to short-chain fatty acids on polymorphonuclear leukocytes and neutrophils (Brunkhorst et al., 1992; Le Poul et al., 2003). The activation of these receptors leads to alterations in the levels of intracellular calcium and cellular motility (Brunkhorst et al., 1992), which may promote the migration of immune cells to areas, such as the digestive tract, where PA levels are high. The latter explanation could be further corroborated when considering a number of *in vitro* studies suggesting that a variety of cells in the gut (Brunkhorst et al., 1992), immune system (Brunkhorst et al., 1992), and CNS (Rorig et al., 1996) can concentrate PA and other weak organic acids, thus leading to intracellular acidification.

The observed increases in the cortex and medulla IFNγ are also in agreement with the work of MacFabe et al. (2007), in which the neurohistological examination of hippocampus and adjacent white matter (external capsule) of PA-treated rats showed an increase in reactive astrogliosis and activated microglia, indicating

Table 1. Estimation of microorganisms recovery in hamster`s intestinal tract before and after treatment with clindamycin antibiotic.

Organisms isolated	Media used and incubation condition	Control hamster	Clindamycin recipient
Staphylococci, group of gram-positive bacilli	MHA/ aerobic: 37°C/24 h	++	+++
Enterobacteriacaea (lactose fermentor)	MCA/aerobic: 37°C/24 h	0	+++
Group β streptococci	BAP/aerobic: 37°C/24 h	+	++
Candida albicans	SDA/aerobic: 25°C/48 h	+	++
Clostridia	CCFA/anaerobic; 37°C/ 72 h	0	++

MHA, muller hinton agar; MCA, Macconkey agar; BAP, 5% sheep blood agar; SDA, sabouroud dextrose agar (yeast media); CCFA, modified cefoxitin cycloserine fructose agar.

Table 2. Mean ±S.D of INF- γ (ng/100 mg), MD (UM/100 mg), and HSP70 (ng/100 mg) levels and GPX activity (U/100 mg), in the cortex and medulla homogenates of hamsters.

Group	Homogenate	Parameter			
		INF- γ	GPX	MD	HSP70
Control	Cortex	90.01 ± 5.14	5.17 ± 0.86	21.91 ± 2.15	32.66 ± 2.53
	Medulla	81.26 ± 1.95	6.11 ± 0.42	18.56 ± 1.43	29.69 ± 2.88
PA	Cortex	141.59 ± 6.28**	2.49 ± 0.32**	46.69 ± 3.06**	57.69 ± 2.57**
	Medulla	98.57 ± 3.49**	3.26 ± 0.40**	36.55 ± 1.91**	59.14 ± 2.38**
Clindamycin	Cortex	84.44 ± 3.55	3.34 ± 0.39**	31.52 ± 1.98**	35.70 ± 1.99
	Medulla	84.09 ± 4.37	3.65 ± 0.54**	21.66 ± 1.41**	34.14 ± 2.17*
Carnosine	Cortex	86.79 ± 1.72	6.59 ± 0.21*	17.64 ± 2.18*	30.90 ± 1.20
	Medulla	83.59 ± 2.56	5.84 ± 0.75	17.72 ± 2.13	31.08 ± 3.46
Carnitine	Cortex	85.88 ± 1.82	7.08 ± 0.18**	18.45 ± 2.07*	31.27 ± 1.95
	Medulla	79.10 ± 3.84	5.62 ± 0.34	16.11 ± 1.16*	33.62 ± 3.85
PA +Carnosine	Cortex	117.07 ± 4.12**	3.39 ± 0.31**	36.98 ± 1.68**	43.66 ± 2.10**
	Medulla	87.94 ± 1.27**	4.03 ± 0.31**	26.19 ± 0.79**	48.19 ± 2.61**
PA +Carnitine	Cortex	119.04 ± 2.79**	3.20 ± 0.31**	37.82 ± 3.77**	44.95 ± 0.94**
	Medulla	88.78 ± 2.46**	4.39 ± 0.42**	28.71 ± 2.12**	44.14 ± 3.68**
Clindamycin+	Cortex	76.51 ± 4.03**	3.61 ± 0.20*	23.95 ± 1.99	35.99 ± 2.17*
Carnosine	Medulla	78.59 ± 4.03	3.56 ± 0.33**	23.96 ± 1.11**	34.03 ± 2.74*
Clindamycin +Carnitine	Cortex	79.49 ± 1.71**	3.42 ± 0.28**	24.16 ± 3.27	37.68 ± 1.38**
	Medulla	83.27 ± 0.71	4.34 ± 0.37**	23.66 ± 1.67**	35.66 ± 1.10**

*Significant at 0.05 level; ** significant at 0.01 level.

that a neuroinflammatory process took place. The same study also proved that the intra-ventricular administration of PA in rats may provide a means to model several aspects of human autism. In the present study, the findings demonstrate elevated levels of IFNγ in the cortex and medulla of PA-treated hamsters. These findings, together with the ones reported by MacFabe et al. (2007) on the modeling of autism in rats administered PA intraventricularly, could find support in the work of Li et al. (2009) where pro-inflammatory cytokines (TNF-α, IL-6), Th1 cytokine (IFN-γ), and chemokine (IL-8) were noted to significantly increase in the brains of autistic patients as compared to healthy controls.

Symbiotic mutualistic interactions between gut microbiota and the host are delineated at several physiological levels. Gut microbiota, generate SCFAs including acetate, propionate, and butyrate which have a variety of physiological and pathophysiological effects. In the present study, while the neurotoxic dose of PA induced significant elevation of INF-γ, the bacterial overgrowth due to clindamycin treatment did not show significant change in this cytokine. This could be attributed to the fact that butyrate as one of the major SCFAs produced has the ability to suppress the secretion of INF-γ as inflammatory cytokine by blocking NF-κB (Segainj et al., 2000).

Table 2 demonstrates that the levels recorded for IFN-γ in the brain cortex and medulla of clindamycin-treated hamsters did not undergo a significant change when compared to those belonging to the control group. This could be attributed to the effects exerted by butyrate as a metabolic product of overgrown bacteria with regards to the suppression of inflammatory cytokines. Since intestinal bacterial overgrowth vary in manifestation, from being only mildly symptomatic to suffering from chronic diarrhea, weight loss, and mal-absorption (Dukowicz et al., 2007), the non-significant decrease recorded in terms of IFN-γ together with the absence of diarrheal signs could help to suggest that, under the experimental conditions used, propionate did not accumulate to a level that could induce pro-inflammatory cytokines.

The accumulation of MD, a lipid peroxidation by-product that has been used as an indicator of cellular oxidation status, is significantly increased in several neurological diseases, such as brain ischemia/reperfusion, Alzheimer's disease, Parkinson's disease, and autism (Al-Gadani et al., 2009) *in vivo*. The data presented in Table 2, Figure 1b and c show the significant decrease of GPX, the increase of MD in PA-treated and clindamycin-induced bacterial overgrowth group of hamsters, and the protective effects of carnosine and carnitine in the four protected groups, respectively. It could be easily observed that the oxidative stress induced by the overgrowth of bacteria was not comparable to that induced by the neurotoxic dose of PA. The significantly high increase in lipid peroxidation together with the significant decrease in GPX activity reported in the present study with regards to the orally administered PA group as compared to controls are in agreement with the previous results of MacFabe et al. (2008) that reported an increase in oxidative stress markers (lipid peroxidation and protein carbonylation) and GSH *S*-transferase activity coupled with a decrease in GSH and GPX activity in the brain homogenates of hamsters to which PA was infused intraventricularly. Furthermore, the relationship between the induction of oxidative stress and the neurotoxicity in PA-treated hamsters find support in the recent work of Cheng et al. (2011) where MD treatment *in vitro* was found to reduce cortical neuronal viability in a time- and dose-dependent way and to simultaneously induce cellular apoptosis as well as necrosis.

The induction of oxidative stress reported for the PA-treated hamsters of the present study could presumably be considered to have occurred via the induction of mitochondrial dysfunction (Brass and Beyerinck, 1988). Other effects could include the sequestration of carnitine (Brass and Beyerinck, 1987) and the increased levels of propionyl coenzyme A, which could result in the inhibition of short chain fatty acid oxidation. Elevated PA could also produce sensitivity to oxidative stress which could, in turn, result in an increase in the damage caused by other environmental toxic factors (metals) or infectious agents (Wajner et al., 2004). The amelioration of induced oxidative stress (increased MD and decreased GPX) through the protection with carnitine prior to the oral administration of the neurotoxic dose of PA (Table 2, Figures 1b, 1c) could help infer that PA induced carnitine depletion among the several mechanisms of PA neurotoxicity, which was also suggested in a previous work by Brass and Beyerinck (Brass and Beyerinck, 1987). This suggestion could be further confirmed when considering the effect of carnitine administration which, in the absence of PA, was effective enough to induce GPX activity and reduce MD levels in the cortex and medulla of treated animals (Table 2, Figures 1b, 1c). In this context, several studies have previously shown that L-carnitine suppresses oxidative damage during aging (Arockiarani and Panneerselvam, 2001). Vanella et al. (2000) reported that L-propionylcarnitine showed a dose-dependent free radical scavenging activity. It was, for instance, shown to scavenge superoxide anion, to inhibit lipoperoxidation of linoleic acid, and to protect against damage induced by H_2O_2 in the presence of ultraviolet radiation (UV).

Furthermore, the neuroprotective effect reported in the present study for carnosine is in line with the recent findings of Cheng et al. (2011) reported on the perfect protection exhibited by carnosine against MD-induced cell injury as well as on its multi-potent potential, including the alleviation of MD-induced protein cross-linking, mitochondrial transmembrane potential ($\Delta\psi m$) decrease, and reactive oxygen species burst. The neuroprotective effect of carnosine could find further support when considering its effects in the absence of PA. In fact, the findings revealed that carnosine showed high potency in the induction of GPX activity and reduction of MD levels in the cortexes and medullas of the hamsters belonging to the untreated and PA and clindamycin-treated groups (Table 2, Figure 1c). A previous experimental work on the protective effects of carnosine that involved a controlled 8-week trial in which 400 mg of carnosine were orally administered twice per day to double-blind placebo reported that the treatment produced a significant improvement in autistic children as compared to the placebo group. Psychometric tests revealed remarkable improvements in vocabulary, socialization, communication, and behavior. Some of the possible physiological mechanisms reported for the carnosine effect in autism included its prevention of NO toxicity (Fontana et al., 2002), the binding of free radicals and reactive hydroperoxides, and the ability to complex with metals, such as copper (Kohen et al., 1988). The copper-carnosine complex was also reported to demonstrate antioxidant, superoxide dismutase (SOD)-like activity *in vitro* (Kohen et al., 1991).

Although the fundamental functions of heat shock proteins (HSP) include molecular chaperoning and cellular repair (Stetler et al., 2010; Plumier et al., 1997), their expression *per se* is a sensitive marker of metabolic

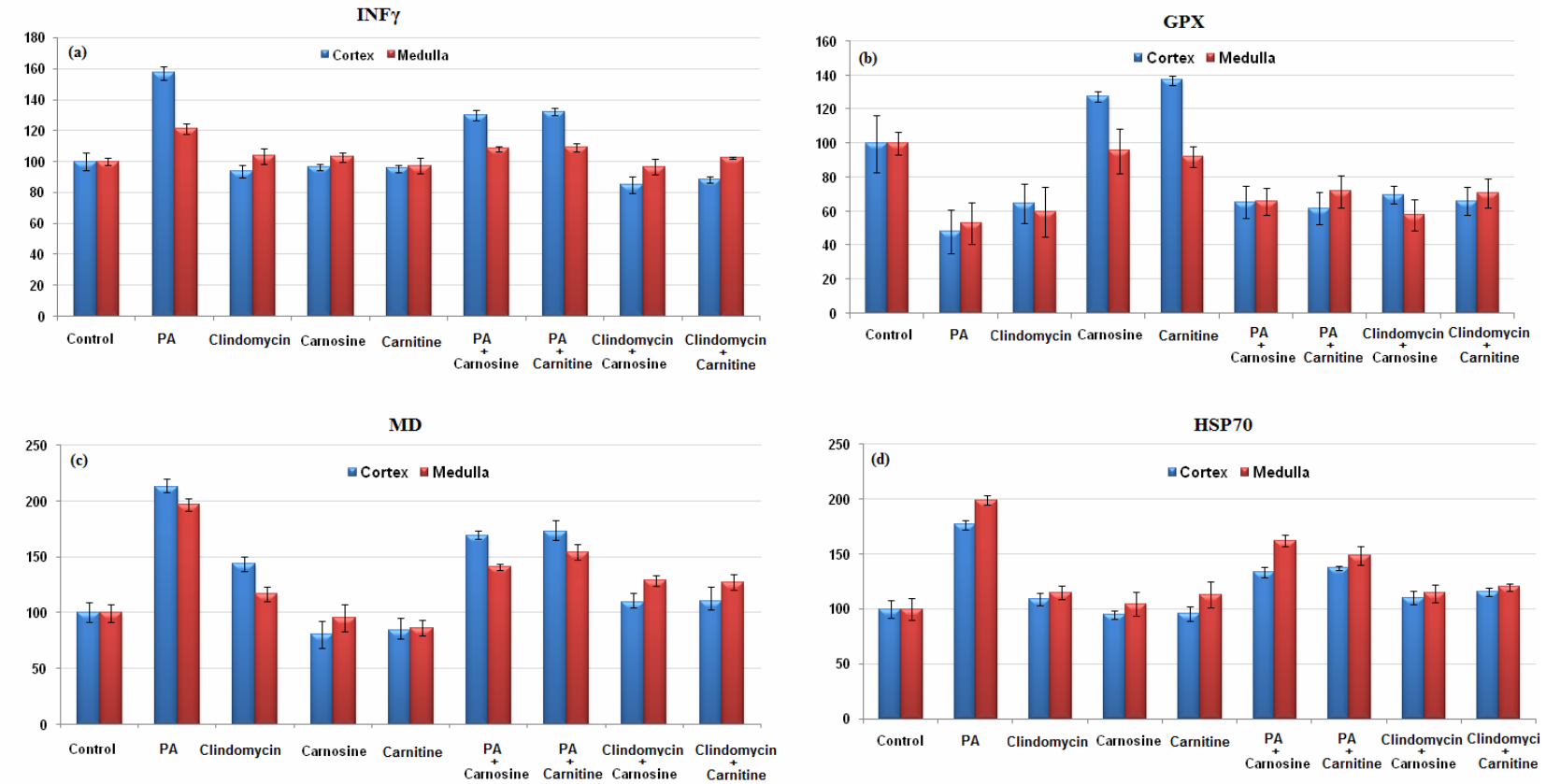

Figure 1. Levels of the parameters measured in the cortex and medulla homogenates of the nine groups of hamsters. Mean ± S.D of INF-γ (a), GPX (b), MD (c), and HSP70 (d) measured in treated hamsters compared to the control groups.

activation or oxidative stress. The induction of brain HSP70 messenger RNA and protein, or both, has been found in quite different situations ranging from psychophysiological stress (Fukudo et al., 1995), heat stress (Bechtold et al., 2000; Westman and Sharma, 1998), intense physical exercise (Lancaster et al., 2004; Oqura et al., 2008), and the use of various psychoactive and addictive drugs (convulsants, amphetamine-like stimulants, morphine, or cocaine) (Sharma and Ali, 2006; Sharma et al., 2009). All these drugs and situations are often reported to increase metabolism and to induce hyperthermia (Kiyatkin, 2010; Kiyatkin, 2005), with the latter factor often being assumed as the driving force for the expression of HSP.

Table 2 and Figure 1d illustrate the significant increase of HSP70 in the cortex and medulla of the PA-treated hamsters and its less significant increase in the brain medulla of the clindamycin-treated group. Moreover, carnitine and carnosine were noted to exert protective effects manifested through their enhancement of HSP 70. The increase of HSP70 could be attributed to the induced neuro-inflammation (IFN-γ) and oxidative stress (MD and GPX) reported in the present study.

Pearson correlations (Figure 2) demonstrated significantly positive associations between IFN-γ, HSP70, and MD but negative correlations

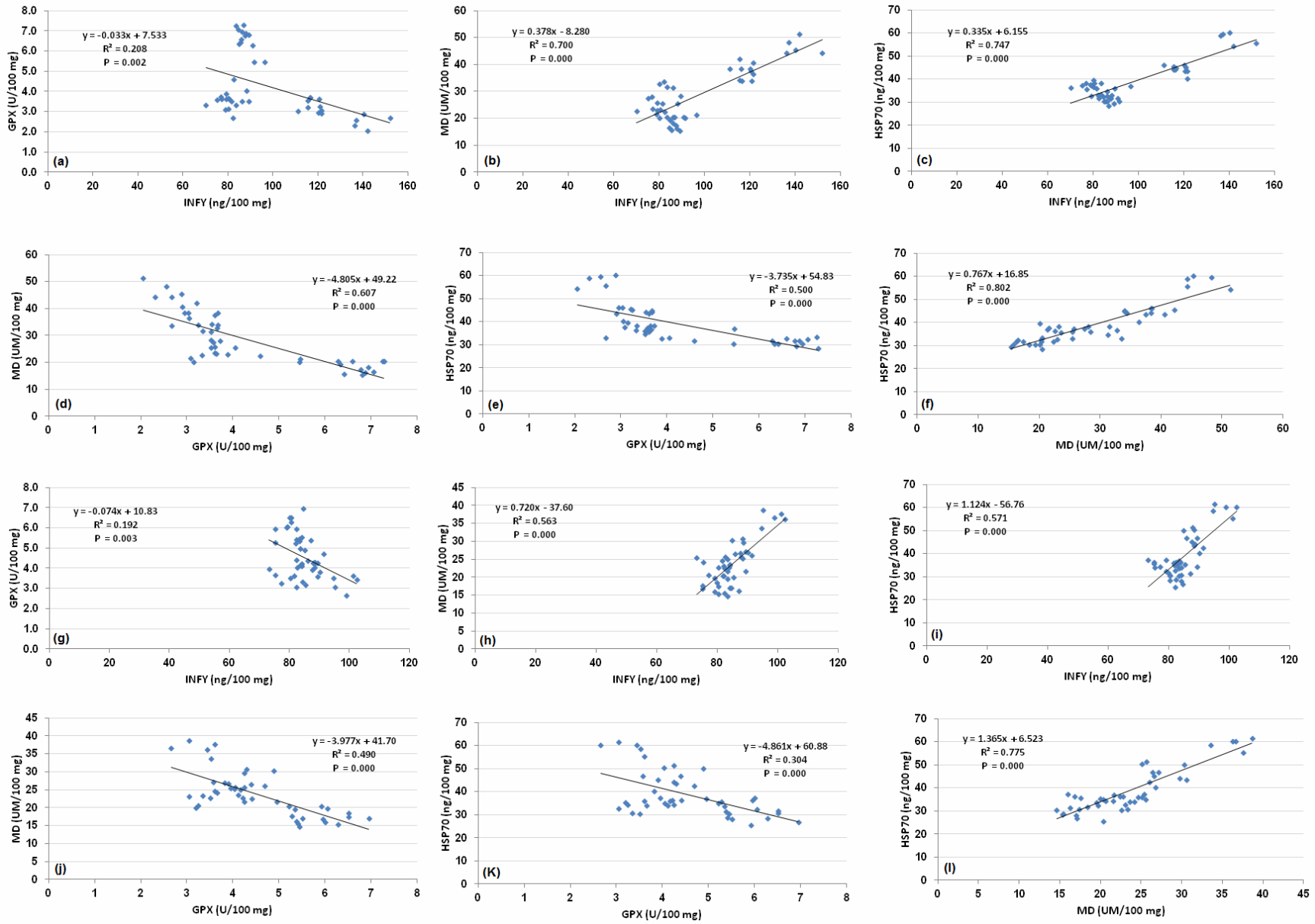

Figure 2. Pearson's correlations of the most significant positive and negative correlated variables with best fit line/curve.

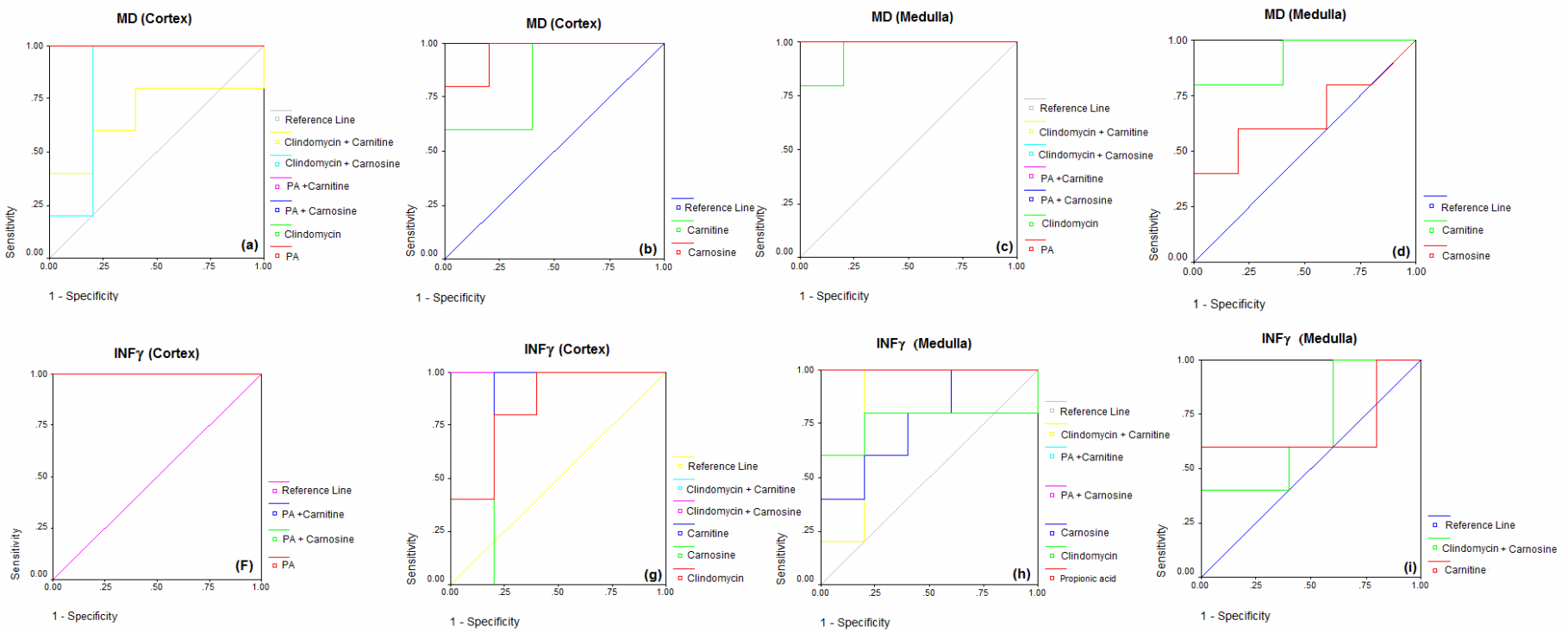

Figure 3. Analysis of receiver operating characteristics of selective parameters measured in the cortex and medulla homogenates of the nine groups of hamsters.

between GPX and MD as well as IFN-γ and HSP70, which could confirm the impairment of the pro-oxidant/antioxidant status in the PA and clindamycin-treated groups of hamsters.

ROC analysis presented in Figure 3 show AUC together with the cut-off values of the four parameters measured for the nine groups under investigation. The total area under the ROC-curve was calculated as a measure of the performance of the treatment either with PA or clindamycin. The area under the curves proved the marked effects of both treatments together with the ameliorative or protective effects of carnosine and carnitine. This could also be supported by the high sensitivity and specificity recorded for IFNγ, GPx, MD, and HSP70.

The neurotoxic effects associated with the orally administered PA presented in the current study, together with the role previously reported for PA (MacFabe et al., 2007; El-Ansary and Al-Daihan, 2011) in the etiology of autism, find support in the recent work of Adams et al. (2011) which declared that children with autism have lower PA concentrations in their stools. This was attributed either to lower levels of beneficial bacteria that produce SCFA's, lower intake of soluble fiber, or much probably to increased absorption due to increased gut permeability. The latter explanation is highly plausible in light of the findings reported by MacFabe et al. (2007) demonstrating that PA can induce autistic-like symptoms when injected into hamsters.

Overall, the findings presented in the current work clearly indicate that although bacterial overgrowth induced by clindamycin was effective to show signs of neuronal toxicity in the cortexes and medullas of the hamsters, its effects were not comparable to those induced by the orally administered dose of PA (250 mg/kg body weight/day

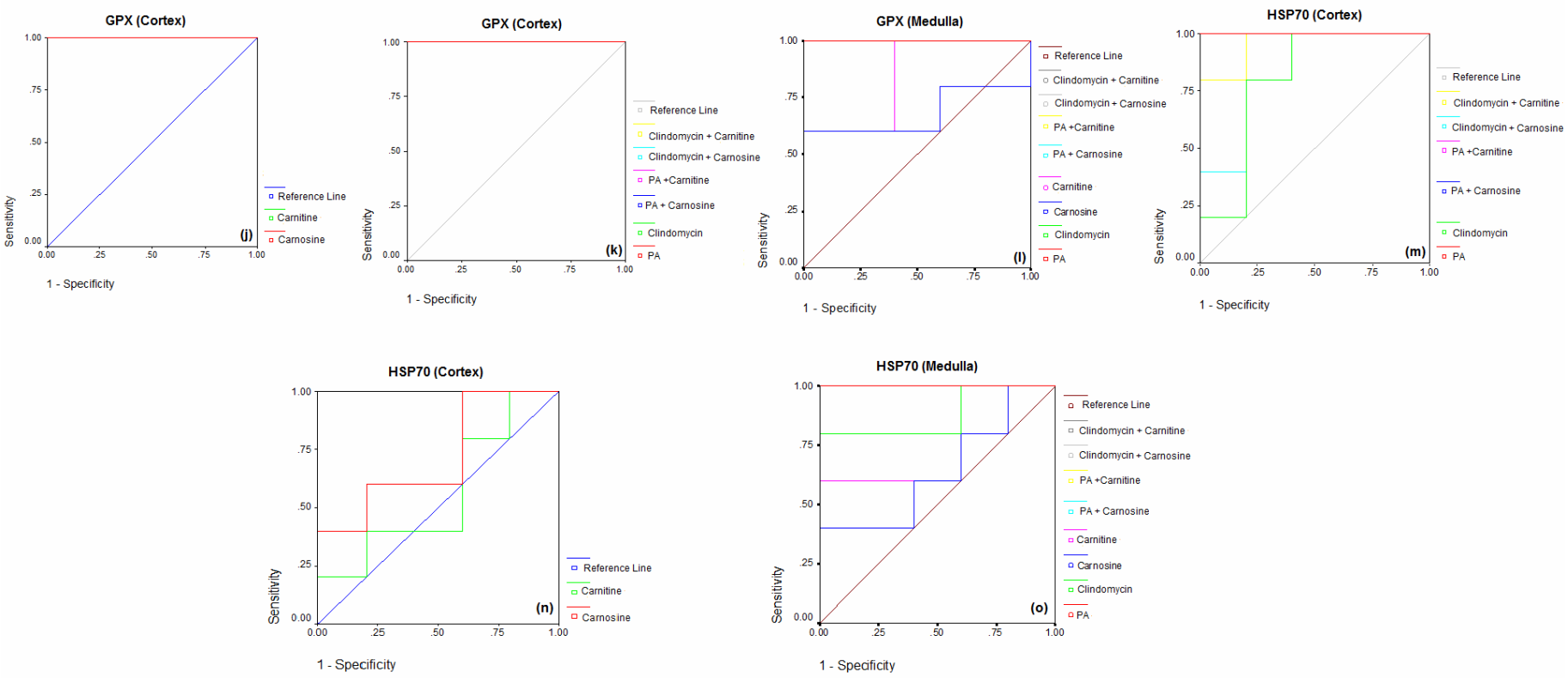

Figure 3. Continued.

for 3 days). This could be attributed to the generated hypothesis stipulating that the overgrowth of specific bacteria might not be enough to manifest significant neurologic harms until there is toxic exposure to a dietary regimes or environmental stressor (Hyman et al., 2006). It is well documented that children with autism have a defect in their biochemical response to oxidative stress (Al-Gadani et al., 2009) which could increase their tendency to accumulate toxic metabolites, such as PA. Nevertheless, in order for such hypotheses to gain empirical evidence, future studies are needed to test the effects of different dietary regimes on the nature of the bacterial overgrowth induced by clindamycin and the neurotoxic effects involved in the process.

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Abbreviations

AUC, Area under the curve; **CFU,** colony-forming unit; **CNS,** central nervous system; **GBA,** gut-brain axis; **GI,** gastrointestinal; **GPX,** glutathione peroxidase; **GSH,** glutathione; **H_2O_2,** hydrogen peroxide; **HSP70,** heat shock protein70; **INF-γ,** Interferonγ; **IL-6,** Interlukin-6; **MD,** malondialdehyde; **NF-κB,** nuclear factor kappaB; **PA,** propionic acid; **ROC,** receiver operating characteristic curve; **SCFAs,** short chain fatty acids; **TNFα,** tumor necrosis factor α.

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