Modulation of heat shock protein immunolocalization in cerebral cortex by melatonin therapy in heat stressed rats

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Abstract
Heat stress leads clinically to manifestations of central nervous system dysfunction. Heat shock proteins (HSPs) function as molecular chaperones protecting the cells from damage. Melatonin has therapeutic and prophylactic effects on heatstroke-induced multiple organ dysfunction. The present work aimed to investigate the protective effect of melatonin against heat stress-induced histological and HSP60 immunoeexpression alterations of cerebral cortex in adult male albino rats. Forty adult male albino rats were used and divided into three groups; I (control) II (3 days heat stressed) and III (3 days heat stressed with melatonin treatment). Cerebral cortex specimens were processed to prepare sections for light microscope examination and blood samples were obtained for oxidative stress and antioxidant analysis. Hematoxylin and eosin stained sections showed degenerative changes in cerebral cortex of rats exposed to heat for 3 days in the form of apoptotic neurons and swelling of astrocytes processes. Neuropil edema, subarachnoid hemorrhage and congested blood vessels were also observed with accompanied increase in serum oxidative stress markers and decrease in antioxidant marker. Melatonin treatment with heat stress showed more preserved nervous tissue with accompanied increase in serum antioxidant marker. HSP60 immunoeexpression was weak cytoplasmic in control group which significantly increased and became strong cytoplasmic after 3 days of heat exposure. With melatonin treatment with heat stress nuclear, HSP60 immunoeexpression was moderate nuclear in some neurons and strong cytoplasmic in others. In conclusion, melatonin ameliorates heat exposure induced degenerative changes in cerebral cortex of adult male albino rats most probably through antioxidant effect with modulation in HSP60 immunoeexpression pattern. Hence, more attention should be thrown to melatonin as a protective treatment to avoid heat stress degenerative effect on cerebral cortex.

Keywords: Microscope, Albino rat, HSP60, Melatonin

Introduction
Temperature has important drawbacks on body fitness and the adaptation to global warming and to the changes in thermal environments is important [1]. In the tropical climate zone, increasing temperatures affecting developing countries disproportionately because it reduces work productivity and hence economic productivity could be diminished [2].

Heat stroke is a life-threatening illness that is characterized clinically by manifestations of central nervous system dysfunction such as delirium, seizures or coma. The most effective therapy is the rapid body cooling and supporting the function of several organs. However, despite these efforts, many patients exposed to permanent neurological disorders, thermoregulatory dysfunction or even death [3]. Heat cytotoxic effect and the systemic inflammatory led to multiple organs damage and heat stroke is better to be prevented than to be treated [4]. Heat stress was reported to disrupt specific and non-specific immune responses in intestinal tract of pigs with barrier disruption [5]. Food consumption and gaining body weight were observed to be decreased after a daily mice exposure to 38.5°C for 60 min for 2 weeks. Also, the levels of corticosterone and vasopressin in the blood, and catecholamine and serotonin metabolite in the hypothalamus increased when mice exposed to above 37°C for 60 minutes [6]. Previous investigators suggested that...
heat stress led to deactivation of autophagy and suppressed autophagosomal degradation that led to accumulation of damaged mitochondria in muscle cells with formation of abnormal cytoplasmic environment [7].

In the cell, the proteins to fulfill their biological functions must be folded as an essential process which is aided by molecular chaperones [8]. Heat shock proteins (HSPs) are the major molecular chaperones whose function is to mediate the proper folding of cell proteins under normal conditions and to maintain their native conformations during stress conditions and also help in refolding of proteins that denatured due to stress [9,10]. Under normal conditions, HSP levels in the cell able to mediate proper folding and match the protein synthesis. However, under cellular stress, synthesized proteins unfold and exceed the capacity of cellular chaperones to prevent protein aggregation leading to loss of protein homeostasis and proteotoxicity [11]. Moreover, HSPs are required for trafficking of protein to its target organelles and to facilitate transfer of misfolded proteins to proteasome for degradation and have role during vertebrate neurogenesis [9,12]. In addition, HSPs are essential to buffer mutations that may disrupt protein's ability to fold [13]. As the name denotes, the expression of HSPs were firstly demonstrated as a response heat shock but were demonstrated also to be induced by other stresses such as, ischemia, hypoxia and heavy metals [14-17].

HSPs manipulation was reported to be involved in cell death signaling and affect the fate of cells in neurological injury and disease states [18].

Melatonin is the main secretion of pineal gland and possesses antioxidant and anti-inflammatory actions [19]. The therapeutic and prophylactic effects of melatonin was previously investigated on heatstroke-induced multiple organ dysfunction syndrome in rats under general anesthesia [20,21].

Hence, the aim of the present work was to investigate the protective effect of melatonin on cerebral cortex against heat stress-induced histological and HSP60 immunopexpression alterations and on serum oxidative status in adult male albino rats.

Material and methods
Forty adult male albino rats (6 months old & 240 gm ±20 body weight) were obtained from the Egyptian Organization for Biological Products and Vaccines. Rats were kept in individual polyethylene cages with stainless-steel tops at the Animal House, Faculty of Medicine, Zagazig University. Before the start of thermal experiments, rats were subjected to controlled conditions of ambient temperature (22-26°C) and relative humidity of 50%–55% and illumination (12h-light/dark), and allowed free access to normal rat chow diet and water ad libitum. The experiment was carried out in compliance with the “Guide of the Care and Use of Laboratory Animals” [22]. Experimental protocols were approved by the ethical committee of the faculty of medicine, Zagazig University. One week after acclimatization rats were randomly divided into three equal groups.

Group I (control group): Included twenty rats were divided into 2 equal subgroups, ten rats each: Subgroup Ia (Negative control) and. Subgroup Ib (melatonin): as rats of subgroup Ia and received melatonin intraperitoneally at a dose of 10 mg/kg body weight [23].

Group II (heat stressed group): Included ten rats exposed to whole body heating (WBH; 41°C and relative humidity 50%–55%) for 1 h daily in an environment-controlled chamber for 3 days. Core temperatures were measured every 1 minute with a copper constant thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo, Japan). Every day, the heat-stressed rats were returned to the normal room temperature (22-26°C) and were properly fed and hydrated after the end of the heat exposure [24].

Group III (heat stressed with melatonin treatment group): Included ten rats exposed to whole body heating as rats of group II but with simultaneous treatment with melatonin intraperitoneally at a dose of 10 mg/kg/ body weight [23]. Melatonin was purchased from Sigma (St Louis, MO, USA).

Histological study
At the end of the experiments, the animals were sacrificed by decapitation under mild anesthesia (anesthetic ether) and the skulls were carefully opened. To prepare paraffin blocks, brains were immediately placed in 10% buffered formalin. After 10 min, when the cerebral tissue was hardened to avoid soft tissue dissipation, 1 cm² specimens were taken from the cerebral cortices of the anterior lobe above the primary fissure. The specimens were fixed in 10% buffered formalin for 24 h and processed to prepare 5 µm sections stained with hematoxylin and eosin (H&E) stain for histological study [25]. For immunohistochemical study, the deparaffinized 5 µm paraffin sections on charged slides were used for localization of heat shock protein (HSP) using avidin–biotin-complex (ABC) immunoperoxidase technique. The sections were incubated in hydrogen peroxide for 10 min to block the endogenous peroxidase then incubated with the primary anti-HSP60 antibody at 1:100 dilutions for 20 min at room temperature. The primary antibody used was a mouse monoclonal antibody HSP60 Ab-1 obtained from Lab Vision Corporation, Medico Co., Egypt (Cat. #MS-120-P0, Clone LK1). Then the slides were washed with phosphate buffer then incubated with the secondary anti-mouse antibodies universal kits obtained from Zymed Corporation. Staining was completed by incubation with substrate chromogen DAB (3,3’ Diaminobenzidine) for 5–10 min which resulted in brown-colored precipitate at the antigen sites and Mayer’s Hematoxylin was used as a counter stain. Positive control was MAD109 cells in breast cancer. For negative controls, incubation was carried out with the omission of the primary antiserum [26].

Sections were viewed using an Olympus microscope (CS060-AUD, SH01155 JAPAN) and images were captured
Biochemical study
Blood samples were obtained by repeated needle puncture of the tail tip veins and put into plain sample bottle for lipid peroxidation and antioxidant analysis. The blood sample was allowed to clot and centrifuged at 4000 g for 10 minutes and the serum obtained was used for the estimation of serum malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and TNF-α.

Malondialdehyde (MDA) levels in samples were measured using the thiobarbituric acid reaction method [27]. The principle of the method based on that MDA can react with thiobarbituric acid (TBA) and give pink-colored trimethine complex with a maximum absorption at 530-532 nm. The samples were analysed by a spectrophotometer. To measure MDA level, 1.25 ml of 20% trichloroacetic acid (TCA) was added to 250 μL serum and shaken in centrifuge tube. To the previous mixture, 500 μL of TBA was added, mixed and placed in boiling water for 10 min. After cooling, 2 ml of n-butanol was added to the mixture, shaken and centrifuged at 3000 rpm for 10 min. Finally, supernatant resultant n-butyl-alcohol layer was separated and the optical density was measured at 535 nm by comparing the absorption to the standard curve of MDA equivalents generated by bisdimethylacetal. The values of MDA were expressed as nmol/L.

Serum TNF-α in individual study groups were determined in serum using ELISA [28]. Determinations were performed according to the manufacturer’s instructions using commercially available kits (Biosource International, Camarillo, CA, USA). Serum samples were added to a 96-well microplate coated with monoclonal antibodies against TNF-α. During incubation at room temperature, TNF-α were immobilised by solid-phase-bound antibodies. Subsequently, the unbound factors were rinsed away and the conjugate bound to a specific enzyme was added to the wells. The wells were rinsed to remove the unbound conjugate part. In order to determine the amount of proteins in the sample, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured at 450 nm is in proportion to the amount of TNF-α bound in the initial step. The sample values are then read off the standard curve. Concentrations were expressed in pg/ml.

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumenehydroperoxide [29]. 100 μL of serum was incubated for 5 minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 μL of cumenehydroperoxide (1 mg /ml), and the rate of disappearance of NADPH with time was determined spectrophotometrically by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of NADPH to NADP per minute.

Morphometric study
The image analyzer computer system Leica Qwin 500, (Cambridge, UK) in the image analyzing unit of the Pathology Department, Faculty of Dentist, Cairo University, Egypt was used. The number of apoptotic neurons was counted in H&E stained sections. The number of HSP60 immunopositive cells was counted and the optical density of nuclear and cytoplasmic HSP60 immunopexpression was evaluated in anti-HSP60 immunostained sections. The measurements were in 10 consecutive fields i from each rat in randomly chosen five animals of each group at a magnification X400. They were measured using the color detect menu and in relation to a standard measuring frame.

Statistical analysis
Data for all groups were expressed as mean ±standard deviation (X±SD). The obtained data were analyzed by SPSS program version 15 (Chicago, USA; “http://WWW.SPSS.com”). Statistical analysis using one-way analysis of variance (ANOVA) test for comparison between different groups was conducted. The results were considered to be statistically significant, highly significant, and non-significant when the P value was less than 0.05, less than 0.001, and more than 0.05, respectively.

Results
The results of subgroups Ia and Ib were similar and did not show any variations; therefore, results of subgroup Ia were represented as the control group (I).

Histological and immunohistochemical results
Light microscopic examination of cerebral cortex of control group showed nerve cells with pale stained nuclei, neuropil and blood vessels surrounded by perivascular astrocyte foot process. Immunohistochemical localization of HSP60 showed weak cytoplasmic HSP60 immunopexpression of nerve cells (Figures 1A and 1B). After 3 days of heat exposure, nerve cells appeared with darkly stained pyknotic nuclei and surrounded by neuropil edema with presence of areas of subarachnoid hemorrhage and congested blood vessel. HSP60 immunopexpression was strong cytoplasmic in the nerve cells (Figures 2A-2C). In heat exposure + melatonin treatment, few nerve cells appeared with darkly stained pyknotic nuclei and few blood vessels were surrounded by swollen astrocytes processes. Some neurons revealed strong cytoplasmic HSP60 immunopexpression and others appeared with moderate nuclear immunopexpression (Figures 3A-3C).

Morphometric results
Statistical analysis the number of apoptotic cells showed significant increase in cerebral cortex of heat stressed group (II) when compared to both control group (I) and heat stressed
with melatonin treatment (III). The number of HSP60 immunopositive cells and the optical density of nuclear HSP60 immunoexpression showed significant increase in cerebral cortex of heat stressed with melatonin treatment group (III) when compared with that of both control (I) and heat stressed group (II). The optical density of cytoplasmic HSP60 immunoexpression was significantly increased in cerebral cortex of heat stressed group (II) when compared with that of both control group (I) and heat stressed with melatonin treatment group (III) (Table 1).

### Biochemical results

Serum MDA and TNF-α showed significant increase in heat stressed group II and non significant increase in heat stressed with melatonin group III when compared with control group I. Serum GPx showed significant decrease in heat stressed group II and non significant increase in heat stressed with melatonin group III when compared with control group I (Table 2).

### Discussion

Hyperthermia was reported to have harmful effects on neuronal structure and function [30]. Neurons, as postmitotic cells, are more vulnerable to the toxic effects of the misfolded and/or aggregated proteins [31]. In the present study, the rats of heat stress group were exposed to whole body heating of 41°C for 1 h daily. The animal housing guidelines recommended maintaining rats between 22-26°C for their normal physiology and development [32].

#### Degenerative changes with heat exposure

Neuropil edema, congested blood vessels and subarachnoid hemorrhage were observed after 3 days of hyperthermia in the present work. That could be explained by previous investigators who observed that hyperthermia above 40°C, in humans, led to increase in cerebral blood flow velocity of about 2 fold and that may increase vascular engorgement with cerebral edema [33]. Moreover, hyperthermia was reported to disturb the cerebral blood flow autoregulation mechanisms leading to disruption of the blood brain barrier integrity and exposing the brain to edema [34].

The present work showed that heat stress led to degenerative changes in cerebral cortex with concomitant significant increase in serum oxidative stress markers MDA and TNF-α and decrease in antioxidant marker GPx. Similar findings were observed after heat stress in neural tissue of mice hypothalamus [35] and in cultured neurons of rat in the form of neuronal apoptosis with shrunken fragmented nuclei with caspase-3 activation and release of cytochrome c [36]. These results are in consistence with previous investigators who reported that heat stress could stimulate programmed cell death of nerve cells leading to brain damage and also exacerbate tissue damage caused by brain trauma, stroke or neurotoxic drugs [37-39].

The cell death due to heat stress could be explained by...
previous investigators who observed that hyperthermia led to impairment of oxidative phosphorylation through the increase in mitochondrial inner membrane permeability with disruption of energy production [40]. Also, exposure of rats to hyperthermia more than 42°C was reported to induce mitochondrial dysfunction in cardiomyocytes [41]. In addition, Riezman suggested that the loss of cell viability, due to heat stress, may be attributed to accumulation of denatured or aggregated proteins [42].

Melatonin ameliorates the degenerative changes
In the present work, simultaneous melatonin treatment with heat stress ameliorated the cerebral cortex degenerative changes with significant decrease in serum oxidative stress markers MDA and TNF-α and increases in antioxidant marker GPx. That was attributed to the reported antioxidant and anti-inflammatory and axonal regrowth-promoting effects of melatonin [24,43]. Previous investigators concluded similar effect of melatonin in decreasing lipid peroxidation markers in brain tissue after thyrotoxicosis in rats [44]. The easy of melatonin crossing through the blood-brain barrier could explain this effectiveness of melatonin in protecting the central nervous system from oxidative damage [45]. Melatonin was suggested as a therapeutic agent against brain edema as it enhances blood brain barrier integrity [46]. Moreover, melatonin was reported to prevent retinal neovascularization, preserved neuroglial function, and attenuated inflammation in the retina of oxygen-induced retinopathy mice via inhibition of vascular endothelial growth factor pathway [47].

Sinha and his colleagues suggested that melatonin neuroprotective effect in hypoxic ischemic brain injury in mouse is mediated by the inhibition of mitochondrial cell death, the restoration of melatonin receptor 1A, and the suppression of astrocytes and microglia [48].

Melatonin acts as a potent nitric oxide scavenger, inhibitor, and/or down regulator of neuronal nitric oxide synthesis and as a mitochondrial metabolism modulator [49]. Melatonin protected against mitochondrial reactive oxygen species-mediated apoptosis in astrocytes [50].

HSP60 immunoexpression after heat exposure
In the present work, exposure to heat led to increase in the immunoexpression for HSP60. Similarly, increased intracellular HSP70 was observed in cultured astrocytes after heat stress [51] and in rat cerebral cortex due to ischemia after occlusion of the middle cerebral artery [52]. Similarly, Dangi and his colleagues observed increased immunocytochemical HSPs localization both in nucleus and cytoplasm of peripheral blood mononuclear cell with heat stress and suggesting a possible role of HSPs on heat stress amelioration [53].

The protective role of increased cellular HSPs after stresses was reported by several previous studies. HSP70 expression was observed by Kim and his colleagues to be increased in optic nerve head and they considered that as a natural cytoprotection against stress [54]. The increased HSPs detected after sublethal heat shock in several organs in rats [57,58] and in hypothalamus of mice [35], could suggest HSPs as a protector against circulatory shock caused by cerebral ischemia and oxidative damage. Previous investigators suggested that

| Table 1. Mean values (±SD) of the number of apoptotic cells and HSP60 immunopositive cells (cells per mm²) and the optical density of cytoplasmic and nuclear HSP60 immunoeexpression among the studied groups. |
|---------------------------------------------------------------|
| Group I (Control) | Group II (Heat stressed) | Group III (Heat stressed + Melatonin) |
|---------------------------------------------------------------|
| Number of apoptotic cells | 10±2.2 | 432±55* | 121±20.1* |
| Number of HSP60 immunopositive cells | 141±12 | 211±17 | 242±21* |
| OD of nuclear HSP60 immunoexpression | 1.3±0.6 | 1.9±0.2 | 9.1±1.8* |
| OD of cytoplasmic HSP60 immunoexpression | 8.2±3.1 | 18.6±4.2* | 10.3±2.4 |

*: Significant difference with group I and III (P < 0.05); #: significant difference with group II (P < 0.05); ± SD: standard deviation.

| Table 2. Mean values (±SD) of MDA, TNF-α and GPx in serum among the studied groups. |
|---------------------------------------------------------------|
| Group I (Control) | Group II (Heat stressed) | Group III (Heat stressed + Melatonin) |
|---------------------------------------------------------------|
| MDA (nmol/L) | 24.5± 2.4 | 39± 4.4* | 26± 3.2 |
| TNF-α (pg/ml) | 23.30± 2.6 | 72.96± 5.6* | 27.2± 3.8 |
| GPx (IU/ml) | 2534.9 ± 71.4 | 1278.1± 51.4* | 2712±73.3 |

*: Significant difference with group I (P < 0.05); ± SD: standard deviation.
increased HSPs, after nerve injury, could protect neurons from apoptotic cell death [59]. Also, it was observed that heat stress led to increased expression of HSPs in the cells of intestinal mucosa that was suggested to improve antioxidant capacity to protect cells from injury [60]. On the other hand, cells deficient in HSPs were reported to be more sensitive to stress with inability to develop thermotolerance [61].

The relation between heat stress and HSPs could be explained by previous investigators who reported that heat shock, as an acute stress condition, cause increase in synthesis of unfolded proteins with abnormal protein aggregation [31,62]. Moreover, thermal stress was reported to result in unfolding of already synthesized proteins but the binding of HSPs to these proteins refolded them and prevented their aggregation [63]. Hence, this increase in HSP60 immunoexpression in present study due to heat stress could be explained by previous investigators who reported that under normal conditions, cellular HSPs levels could match all the level of synthesized protein and mediate their proper folding but under stress, some HSPs expression increased and the newly formed HSPs act as chaperone that bind to denatured proteins trying to restore protein homeostasis and prevent their aggregation [11,64]. The balance between HSPs capacity and the misfolded proteins formation was suggested to be an effective therapy [63]. Barreto and his colleagues observed that HSP72 overexpression after cerebral ischemia was associated with reduced glial fibrillar acidic protein (GFAP) density and hence, suggested modulating effect of HSPs on astrocytes [65]. In addition, overexpression of HSPs was reported to protect brain against ischemia by anti-inflammatory mechanism, through inhibition of innate immune response and several drugs nowadays has been used to induce cellular HSPs through increasing its transcriptional level [66]. Moreover, HSPs were reported to affect the function of mitochondria in the cells of skeletal muscle, adipose tissue and pancreas that suggesting the use of HSPs in diabetes mellitus treatment [67]. On the other hand, enhanced HSPs expression and its extracellular release was reported to act as danger signal in atherosclerosis due to stimulation of immune reactions [17] and cancer cells was documented to be dependent on HSPs and so, HSPs inhibitors could be used as anticancer drugs [63,68].

**HSP60 localization after heat stress and modulatory effect with melatonin treatment**

In the present work, HSP60 immunoexpression in neurons was weak cytoplasmic in control group then was translocated to be strong cytoplasmic in heat stressed group then moderate nuclear and strong cytoplasmic in heat stress with melatonin treatment group. Similarly, HSPs were reported by previous investigators to be cytoplasmic proteins then translocated to the nucleus under cellular stress and growth [69]. That could be explained by previous investigators who reported that normally, HSPs are inactive and bound to heat shock factor (HSF) in the cytoplasm and under heat stress, HSF separate from HSPs and enter the nucleus to bind with heat shock elements (HSEs) in the HSP gene promoter region [57,70]. Then, HSP mRNA is transcribed and leaves the nucleus to the cytoplasm where new HSPs are synthesized with increased cytoplasmic expression. Also, acute exposure of astrocytes to ethanol was observed to stimulate translocation of HSP into the nucleus, to start the activation of HSPs transcription with HSPs accumulation in the cytoplasm [71].

In the present work, melatonin led to significant increase in the nuclear HSP60 immunoexpression in preserved nerve cells after heat stress. That is in consistence with Shi and his colleagues who suggested direct link between melatonin and thermotolerance in Arabidopsis [72]. Also, it was suggested that melatonin prevent the development of pancreatic inflammation after acute pancreatitis through increasing production of HSP60 [73].

**The relation between HSP60 immunoexpression and degenerative changes**

The increased nuclear immunoexpression of HSP60 in the present study was associated with decrease in the number of apoptotic cells with melatonin therapy. Similarly, dramatic increase in nuclear HSP72 was observed in cultured HeLa cells after hyperthermia that was suggested to protect the nucleus against apoptosis [74]. Moreover, they added that the flavonoids such as quercetin are apoptotic inducer through suppression of both HSP72 expression and migration to the nucleus. Also, it was concluded that cytoplasmic HSP27 expression inhibited nuclear apoptosis [75] which was reported to be through binding with cytochrome c and procaspase-3 [76]. In cultured cortical neurons and astrocytes of rat, HSPs were induced 24 hours after heat stress with early translocation of it into the nucleus of astrocytes but that was delayed in neurons. Hence, it was suggested that the more susceptibility of neurons to injury may be attributed to this delay in HSP70 translocation [77]. Previous investigators observed that oxidative stress caused by several environmental stresses lead to translocation of cytoplasmic HSP to the nucleus to protect the cell from damage [15,78]. Moreover, nuclear HSP60 localization was reported to be crucial for stem cells to escape from senescence and improve self-renewal and ability to differentiate into neurons [79]. In addition, Zhang and his colleagues suggested that the regulation of HSP60 production is a potential therapeutic option for the treatment of neurodegenerative disorders [80].

**Conclusion**

Melatonin ameliorates heat exposure induced degenerative changes in cerebral cortex of adult male albino rats most probably through antioxidant effect with modulation in HSP60 immunolocalization. Hence, more attention should be thrown to melatonin and HSPs modulators as a protective
therapy against heat stress.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions


table

| Authors’ contributions | HEH | AAF |
|------------------------|-----|-----|
| Research concept and design | ✓   | ✓   |
| Collection and/or assembly of data | ✓   | --  |
| Data analysis and interpretation | ✓   | ✓   |
| Writing the article | ✓   | ✓   |
| Critical revision of the article | ✓   | --  |
| Final approval of article | ✓   | ✓   |
| Statistical analysis | --  | ✓   |

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