



Cardiotoxicity testing of diglycolic acid using *In Vitro* and *In Vivo* models

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Abstract

Background: Renal and hepatotoxicity of diglycolic acid (DGA) has been described with *in vitro* cellular models as well as *in vivo* animal and human systems. The possibility of DGA being toxic to other organs, such as the heart, has not been well investigated. A human case report identified the heart as a potential target organ of DGA, but an in-house *in vivo* rat study neither found gross nor microscopic pathological changes following repeated oral DGA exposure.

Methods: To better understand the potential of DGA to adversely affect the heart, we focused our current study on evaluating cardiac effects of DGA by treating rat H9c2 cardiomyocytes and human induced pluripotent stem cells (iPSCs) that were differentiated into beating cardiomyocytes (iCells) with increasing concentrations of DGA in cell culture media. We investigated the effects, mechanisms, and value of our *in vitro* cellular cardiac models.

Results: We measured mild effects of DGA on cytotoxicity and the cellular production of reactive oxygen species in H9c2 cells. Cardiomyocyte beat rate (BPM) was also mildly affected by transient treatment with DGA.

Conclusions: Our study indicates that DGA cardiotoxicity *in vitro* correlates with the preliminary findings in our *in vivo* study where rats treated with daily doses of up to 300 mg/kg of DGA orally for 5 days did not show any major signs of abnormal cardiac pathology.

Keywords: Diglycolic acid, heart, cardiotoxicity, *in vitro*

Introduction

Filler or thickener compounds present in dietary supplements or other foods often include carboxymethyl starches, such as carboxymethyl cellulose (CMC) [1]. Interestingly, it has been found that during the chemical synthesis of CMC, a byproduct called diglycolic acid (DGA) is also made and that DGA is a difficult impurity to eliminate [2,3]. DGA is a small four-carbon molecule consisting of two carboxylic acids connected together by an ether linkage (PubChem CID: 8088). The safety of DGA as an indirect food additive is the focus of our study. Mass poisoning incidents caused by unintentional ingestion of diethylene glycol have been recently demonstrated to be caused by its metabolic byproduct, DGA [4]. Although the consumption of DGA as a food additive has not been linked to any adverse event, its widespread presence led us to question its potential

toxicity. In the absence of published *in vivo* dose-response and lethal dose (LD)50 data on DGA, we sought out to begin investigating the potential for DGA to engender toxicity. Human *in vitro* and rat *in vivo* studies by our group and others have established that DGA is an acute renal and hepatic toxin, with the potential of also being a cardiotoxin [5-10]. In a tragic case report of unintentional human DGA oral exposure, not only were the kidneys and liver severely damaged, but there was some evidence of heart injury as well [9]. Our rat *in vivo* study, for example, revealed that after daily gavage dosing of different concentrations of DGA ranging up to 300 mg/kg BW, creatine kinase levels doubled or tripled in the animals that were treated with high doses of DGA, but no direct findings of gross or microscopic cardiac pathology [10]. These preliminary findings may suggest the possibility that the heart is vulnerable

to the primary or secondary effects of DGA. Furthermore, the possibility of DGA-induced cardiotoxicity is also supported by recent findings that human cardiomyocytes express molecular transporters of organic anions including dicarboxylic acids [11,12]. As a dicarboxylic acid, DGA has the ability to chelate divalent cations [13], which could be a mechanism to behave as a possible toxin. Based on these reports therefore, we began an investigation into the potential cardiotoxicity of DGA. Our cardiotoxicity study of DGA aimed to bring together two *in vitro* cellular models using the established rat-derived cell line, H9c2, as well as the newer human induced pluripotent cell (iPSC)-derived cardiomyocytes (iCells), which actually beat *in vitro*. Having achieved its aim, this study contributes to the *in vitro* toxicity profile of DGA in a cardiac model and even affords us the opportunity to assess the level of concordance between our *in vitro* rat and human cellular findings with those of our *in vivo* rat and others' *in vivo* human data. The strength of any laboratory model of human outcomes will ultimately be contingent upon its ability to predict human clinical data.

Materials and methods

Cell culture and treatments

Two types of cells were used in our study. First type was H9c2 rat cardiomyoblast cell line (ATTC, Manassas, VA), whose cells were initially seeded into T75 flasks and expanded. The initial growth media was DMEM (high glucose) with 4 mM L-glutamine, 110 mg/L sodium pyruvate, 10% FBS and 1% antibiotic/antimycotic solution. All components were obtained from Life Technologies (Frederick, MD, USA), except for FBS, which was obtained from Thermo-Fisher (Waltham, MA, USA). After propagation in growth media, cells were removed with by gentle treatment with a trypsin solution and plated at 2×10^5 cells per ml in either white or black 96-well plates with transparent bottoms. On the day after plating, the cells were differentiated by replacing the growth media with differentiation media. The differentiation media was the same composition as growth media, with 2% adult goat serum (Thermo-Fisher) replacing FBS and addition of 20 nM each of all-trans and 9-cis retinoic acids. Cells were grown for seven days in differentiation media (changed every two days) prior to use in cell viability assays.

The second type of cellular *in vitro* model used was iCell cardiomyocytes (Cellular Dynamics, Inc., Madison, WI, USA). Human induced pluripotent stem cell (iPSC) cardiomyocytes (iCell cardiomyocytes) were used to produce a 96-well plate of synchronously beating heart cells. The production of the cells is a proprietary process, but has been described in general terms elsewhere [14,15]. Cardiomyocytes were initially seeded at a density of 2×10^5 cells per ml into gelatin-coated black 96-well plates with transparent bottoms. The different media preparations for plating and subsequent maintenance were provided by Cellular Dynamics. Cells were placed in a humidified air incubator with 5% CO₂ at 37°C. After 48 h, the surface of the cell layer within the wells was gently washed by slowly

aspirating media up and down with a multichannel pipette to suspend non-adherent cells. Plating media was removed, and replaced with warm maintenance media. Maintenance media was changed every other day. Cells were maintained for 10 to 15 days post-plating, which was usually the time required for the cells to beat synchronously in all of the wells.

Cytotoxicity and reactive oxygen species evaluation in H9c2 cells

H9c2 cells were used to screen the test compounds for overt cytotoxicity, prior to their use in beating cells. Cells were exposed to concentrations of DGA from 0.5 to 10 mM for a 24 hour period, similar to our other studies [7,8,16,17]. Cytotoxicity was assessed using a kit for ATP content (Promega, Madison, WI, USA) according to the manufacturer's instructions, except for prior rinsing of wells to avoid interference of the test compounds with light production [16]. Cytotoxicity was also measured in some iCell experiments, after 30 minutes of exposure to DGA at the same range of concentrations.

The production of reactive oxygen species following H9c2 treatment with DGA was assayed using the Promega luminescence system. Following the manufacturer's instructions, cells treated with DGA or Doxorubicin were exposed to a hydrogen peroxide substrate during the last 5 hours of their total treatment (24 hrs) followed by 'Detection Reagent' for approximately 20 minutes. All 96-well assay plates were analyzed by plate reader (BMG Fluorostar by BMG Labtech, Ortenberg, Germany).

Measurement of pH of media plus DGA

DGA was obtained from Sigma-Aldrich and was reported to be of 98% purity. After reading the plates with the FLIPR Tetra instrument to evaluate cell beating (see below), the media was removed and pooled from six wells which received the same concentration of DGA. Media was frozen in a -80°C freezer. About seven days later, it was thawed and heated to 37°C in a water bath placed in the cell culture incubator with a CO₂ level of 4.9 to 5.0%, simulating the conditions under which the cells were tested. The pH was measured with a Fisher Orion Star LogR pH meter (Fisher scientific) equipped with a Ross electrode (Fisher).

Beat rate measurement

Both standard DGA and neutralized DGA were used to study cytotoxicity and effects on beat rate in iCells. A 100 mM aqueous solution of DGA was prepared, then diluted to 0.5 mM, 1 mM, 2.5 mM, 5 mM, 7.5 mM, and 10 mM for experimental use. In the case of neutralized DGA, the 100 uM stock was titrated to the pH of a 50:50 mixture of the media and the EarlyTox calcium detection dye (Molecular Devices) then diluted as above. A FLIPR Tetra instrument (Molecular Devices LLC, Sunnyvale, CA, USA) was used for all measurements with the beating cells. The FLIPR Tetra is a rapid fluorescence plate reader, capable of taking rapid sequential exposures of all wells of a 96-well

plate simultaneously. Signal detection was based on changes in fluorescence, measured with an excitation wavelength of 470-495 nm, and an emission wavelength of 515-575 nm. The principle of the instrument is based on the binding of calcium to a fluorescent dye once it enters the cytoplasm from extracellular sources and the sarcoplasmic reticulum. As calcium increases, a parallel increase in fluorescence occurs, which then declines back to baseline with subsequent reduction in intracellular calcium concentration. Calcium movement corresponds to heart cell contraction, each peak representing one beat. Software supplied with the instrument was used to calculate beats per minute (BPM). In the case of very slow beating, the beat rate was determined manually by counting the peaks present over the two minute sampling interval.

After a pre-determined two hour incubation with a calcium binding dye, 96-well plates of beating iCells were placed in the FLIPR Tetra instrument for reading. After baseline readings were obtained, the cells were returned to the incubator for 15 min. Test compounds were then added, utilizing the automated pipette in the FLIPR Tetra to add the compounds to all 96-wells simultaneously. Cells were then returned to the incubator for 30 min, based on use of this exposure interval in a study of iCell cardiomyocytes exposed to pharmaceuticals [18]. Following the 30 min exposure for treatment, plates were read again to measure the effect of the compounds. All experiments were repeated 2 to 3 times.

Because of differing beat rates pre-treatment, all wells were normalized by dividing the post-treatment value by the pre-treatment value to yield a ratio. All values from a plate were standardized by division by the mean control value for that plate. Data from all plates were then combined for analysis. This was allowable because of the similarity among standardized means for each treatment on multiple plates. Statistical analysis was done using Microsoft Excel and GraphPad software (GraphPad, Inc.).

Results

DGA is cytotoxic to H9c2 cardiomyocytes *in vitro*

To evaluate the potential cytotoxicity of DGA to cardiomyocytes, we exposed H9c2 cells to increasing doses of either DGA or the control cardiotoxin, doxorubicin, for 24 hours *in vitro*. As expected, doxorubicin treatments lead to a significant decrease in H9c2 viability starting with the low exposure dose of 0.5 μM ($P < 0.05$, **Figure 1a**). Relative to this strong toxicity exhibited by doxorubicin, DGA did not significantly compromise cell viability until the exposure dose reached 7.5 mM ($P < 0.05$), which is a dose ~15,000-fold higher than that of first marked toxic dose of doxorubicin in this series of experiments and likely worked with varying mechanisms to produce these different results.

To corroborate these findings, we next quantified the levels of reactive oxygen species (ROS) in H9c2 cultures exposed to DGA or doxorubicin. Just as the two compounds produced varying degrees of cytotoxic effects on cardiomyocytes *in vitro*, they also appeared to exert these effects by different mechanisms of action. *In vitro* treatment with DGA led to significantly higher bursts of ROS production ($P < 0.05$) at the same treatment concentrations that also significantly decreased H9c2 cell viability (**Figure 1b**). Doxorubicin, however, did not significantly affect ROS production at any of the concentrations in the 0-10 μM range tested, despite the finding that this whole range of treatment doses strongly reduced cell viability.

DGA cytotoxicity is not quenched by pH neutralization

We hypothesized that DGA may potentially yield cardiomyocyte toxicity *in vitro* by virtue of its acidic nature. Although cell culture media is routinely buffered, its buffering capacity may have been exceeded by DGA. As shown in **Figure 2a**, measuring the pH value of each DGA treatment concentration in the 0-10mM range showed that the buffering capacity of cell culture media is indeed exceeded at the 5 mM dose and

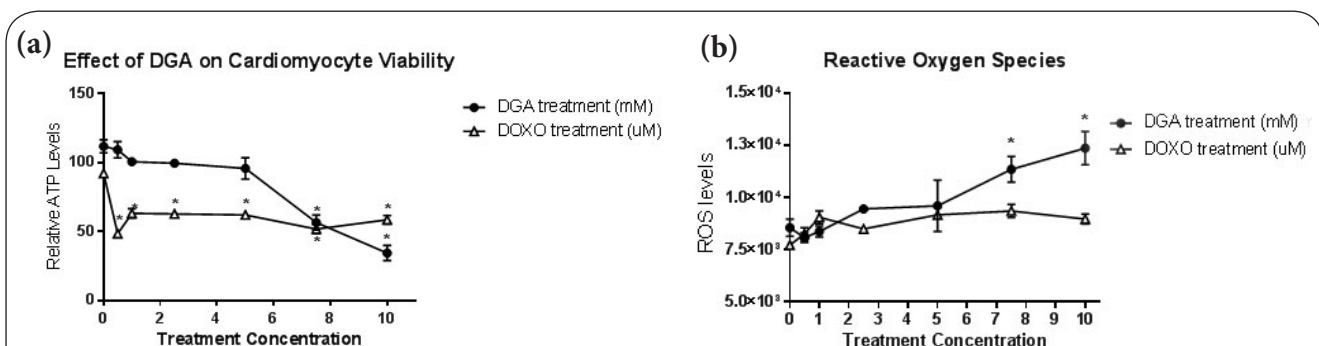


Figure 1. The effect of DGA on cardiomyocyte viability and ROS production. H9c2 cells were exposed for 24 hours to either DGA at concentrations ranging from 0 to 10 mM (filled circles) or doxorubicin at the lower concentration range of 0 to 10 μM (open triangles). (a) Cellular ATP levels of treated cells were normalized to those of untreated cells following assessment by luminescence assay. (b) Cellular ROS levels were also measured by luminescence assay following DGA or doxorubicin treatment. Graphs shown include data from at least 3 independent experiments. *, $P < 0.05$, DGA or doxorubicin treatment vs. no treatment.

beyond ($P < 0.05$). Interestingly, the pH values within this treatment testing range remained above 7.

Given the statistically significant decrease in pH that DGA could produce in cell culture media, we tested whether neutralizing the acidity of DGA could reduce its cytotoxic potential. Surprisingly, we found that if the pH levels of DGA-treated cell cultures were kept at the same levels as those of 'no DGA' treatment controls (pH ~ 7.8), then there was no effect on DGA-induced cytotoxicity. As shown in **Figure 2b**, the trend in cell viability reduction following treatment with DGA or neutralized DGA were very similar.

Effect of DGA on frequency of beating is pH-dependent

Although maintaining the culture media pH around 7.8 did not appear to affect the ability of DGA to induce cytotoxicity in H9c2 cells, we sought to test whether DGA could instead affect the beating capability of cardiomyocytes. Using iCells as our beating cardiomyocyte *in vitro* cellular model, we tested this hypothesis. In the absence of pH-neutralizing intervention, we treated iCells with DGA in the treatment dose range of 0-10mM and measured the frequency of cell beating after 30 minutes of DGA exposure. As a control for beat rate inten-

sification, we used 10 μM isoproterenol and as a negative (no effect) control, we used 50 μM amoxicillin [19]. As expected, isoproterenol induced an increase in beating frequency and amoxicillin did not affect beating. By contrast, DGA treatment was associated with a sharp increase in beating at the exposure dose of 2.5 mM, followed by a complete cessation of beating over the 2.5 mM dose (**Figure 3a**). In the presence of neutralized DGA treatments in the same 0-10mM concentration range, iCell beating frequency increased mildly until cells were exposed to 7.5 mM of neutralized DGA ($P < 0.05$). At 7.5 and 10 mM, the frequency of iCell beating was significantly reduced to about 55% of control (no DGA treatment), as shown in **Figure 3b** ($P < 0.05$).

Discussion

The identification and characterization of DGA as a renal and hepatic toxicant has been unequivocally established using *in vitro* cellular, *in vivo* rat, and even human systems [3,7-10]. A single case report of human exposure indicated that the heart could be negatively affected by DGA, but this effect has never been previously captured *in vitro* or *in vivo* animal models. Given the lack of data on the effect of DGA on the

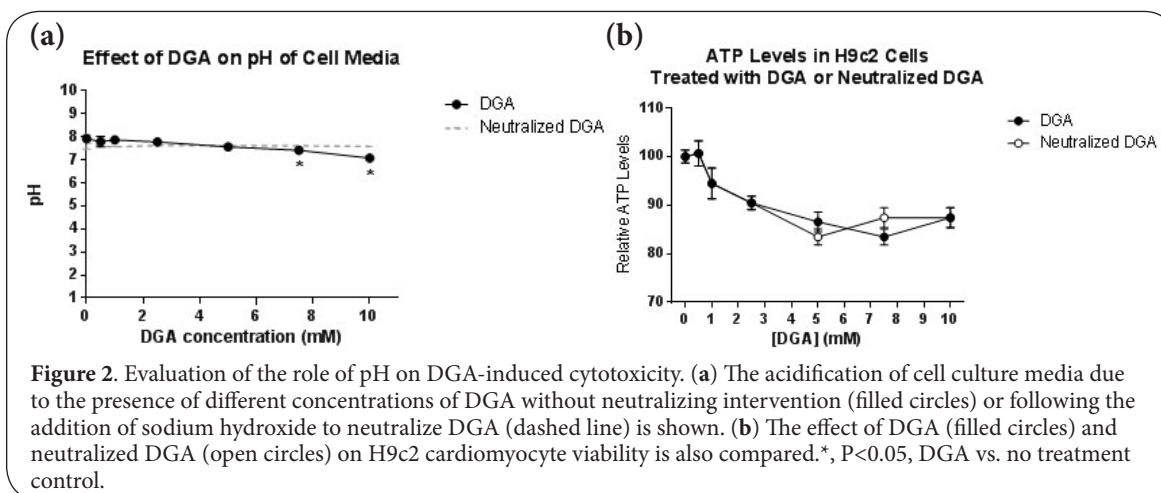


Figure 2. Evaluation of the role of pH on DGA-induced cytotoxicity. (a) The acidification of cell culture media due to the presence of different concentrations of DGA without neutralizing intervention (filled circles) or following the addition of sodium hydroxide to neutralize DGA (dashed line) is shown. (b) The effect of DGA (filled circles) and neutralized DGA (open circles) on H9c2 cardiomyocyte viability is also compared. *, $P < 0.05$, DGA vs. no treatment control.

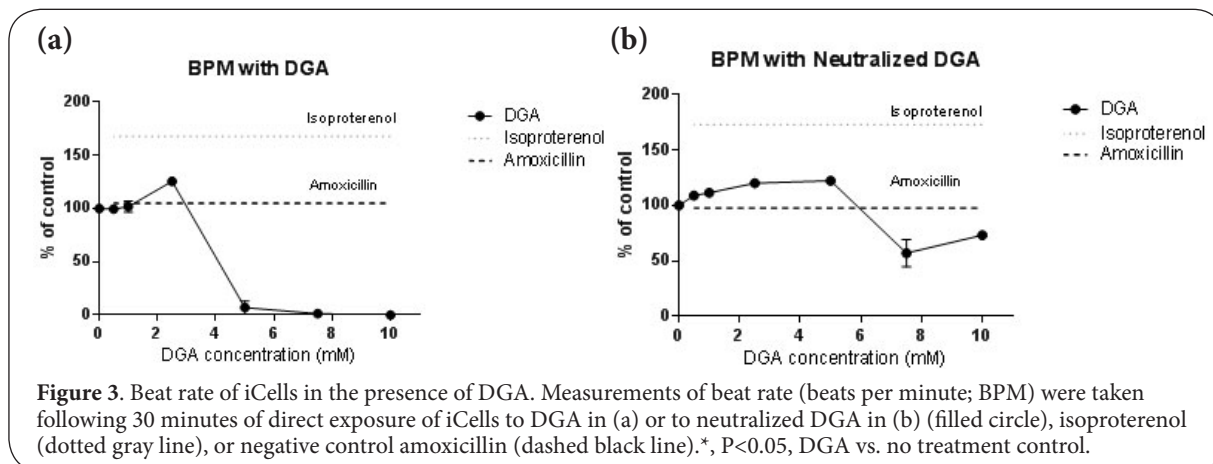


Figure 3. Beat rate of iCells in the presence of DGA. Measurements of beat rate (beats per minute; BPM) were taken following 30 minutes of direct exposure of iCells to DGA in (a) or to neutralized DGA in (b) (filled circle), isoproterenol (dotted gray line), or negative control amoxicillin (dashed black line). *, $P < 0.05$, DGA vs. no treatment control.

heart, we sought to model its potential toxicity using two types of *in vitro* cellular models. First, for the assessment of cell viability as a fundamental evaluation of cellular toxicity, we used H9c2 cells, which is a frequently used cardiac *in vitro* cellular model. The H9c2 cell line is derived from embryonic rat heart tissue and has been validated as a reliable model for its ability to mimic development and disease states [20]. Using this model, we uncovered the potential for DGA to decrease cell viability. This effect was considerably less potent than that of control cardiotoxin doxorubicin and may have worked through a different mechanism of cytotoxicity that involves mitochondrial poisoning through ROS surge production. Although we considered the possibility that the acidifying effect of DGA was primarily responsible for its mode of cytotoxic action, but further investigation challenged this simple concept. By using a second *in vitro* cellular model using human-derived induced pluripotent stem cell (iPSC), we could generate beating iCell cardiomyocytes *in vitro* and assess the effect of DGA and its acidity on beat rate. The use of iPSCs is becoming a popular approach to modeling sophisticated heart functions *in vitro*. The differentiation pathway of iPSCs to beating cardiomyocytes is well established and extremely useful in an *in vitro* setting. In our experience, the beat rate of cardiomyocytes was not only reduced to zero by DGA past the threshold dose of 5mM, but revealed that the role of DGA's acidic properties was partly responsible for its effect on beat rate. Once the cell culture medium was prepared to have the same pH as its control medium lacking DGA, we measured attenuation of beat rate reduction. Clearly, features other than DGA's acidity accounted for its apparent beat rate toxicity, since the reduced frequency remained around 50% following its pH neutralization. In addition to changes in beating rate, we also observed that DGA could negatively affect Ca²⁺ signal amplitude of the beating iCells (data not shown), which is consistent with DGA's ability to chelate divalent metal ions as an additional potential mechanism of toxicity as it has been described in the renal system [21].

By investigating the *in vitro* effects of DGA, we gained the opportunity to compare our findings to that of a recent *in vivo* rat study that was also recently performed by members of our research group [10]. In that study, rats were orally dosed on a daily basis with DGA dose solution ranging in strength from 0 to 300 mg/kg BW for a total of up to 30 days. There in, organ weights were measured to help indicate organ abnormalities and it was found that heart weights were not significantly different in any cohort. The levels of creatine kinase, however,

were about 2- to 3-fold higher in rats in the two highest dose groups (100 and 300 mg/kg BW) compared to levels in rats in the vehicle control group. Because creatine kinase activity can reflect both heart and skeletal muscle injury, it is not an exclusive heart-specific biomarker of toxicity, making it difficult to firmly conclude that the measured elevations were due to DGA effects on the heart. However, in light of the case report describing a man who accidentally ingested DGA and was eventually found to have heart problems [9], it might be reasonable to suspect a connection between DGA poisoning and heart disturbances. The reported heart abnormalities included petechiae, slight ventricular hypertrophy, areas of fibrosis, as well as infiltration by interstitial fat, lymphocytes and other cells. It should be noted that the DGA-to-heart connection may also be secondary to the kidney and liver toxicities that appear to be so profoundly injurious. For example, rising levels of serum potassium due to renal dysfunction might have caused the terminal cardiac arrest described in this case report.

Conclusions

Clearly, the role of DGA in mediating cardiotoxicity has yet to be fully explored, but our study points to the possibility that it may not only compromise cell viability but also affect the beat rate of cardiomyocytes. Reports demonstrating the expression of organic anion transporters on cardiac cells have emerged [11,12] and support the idea that DGA could exert a direct effect on heart cells in addition to possibly affecting heart tissue through secondary mechanisms following renal insufficiency and liver injury. The concordance between the data collected from our *in vitro* model with our *in vivo* rat model reflects the potential strength of *in vitro* systems to forecast *in vivo* animal model results. The added concordance between these two systems and the unique human case report further supports the utility of both rat and human *in vitro* cellular model systems in predicting human outcomes of toxin-mediated organ injury.

List of abbreviations

DGA: Diglycolic acid
 iPSCs: Induced pluripotent stem cells
 BMP: Beats per minute

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	RC	MEM	KB	HT	SV	ZK	VT	TB	NO	AD	KB	JS	PLW	RLS
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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