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Toxicity Assessment of Gallic Acid Loaded Graphene Oxide (GAGO) Nano-Formulation in Zebrafish (*Danio Rerio*) Embryos

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ABSTRACT

Gallic acid (GA) is a phenolic compound found in almost all plants and has been reported to possess powerful health benefits such as anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic properties. However, GA suffers a short half-life when administered *in vivo*. Recent studies have employed graphene oxide (GO), a biocompatible and cost-effective graphene derivative, as a nanocarrier for GA. However, the toxicity effect of this formulated nano-compound has not been fully studied. Thus, the present study aims to evaluate the toxicity and teratogenicity of GA loaded GO (GAGO) against zebrafish embryogenesis to further advance the development of GA as a therapeutic agent. GAGO was exposed to zebrafish embryos ($n \geq 10$; 24hr post fertilization (hpf)) at different concentrations (0-500 µg/ml). The development of zebrafish was observed and recorded twice daily for four days. The toxicity of pure GO and GA was also observed at similar concentrations. Distilled water was used as control throughout the experiment. A significantly high mortality rate, delayed hatching rate and low heartbeat were recorded in embryos exposed to GO at concentrations of ≥ 150 µg/ml at 48 hr ($p<0.01$), 72 hr ($p<0.001$) and 96 hr ($p<0.0001$) post-

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exposure. Interestingly, all measured parameters were significantly improved in embryos exposed to the same concentration of GAGO (100-150 μg/ml), which was comparable to control group at all-time points. The present data demonstrated that GAGO is safe to be used at low concentration exposure (0-150 μg/ml), but further study has to be conducted to correlate the toxicity of GAGO with its effective concentration in *in vitro* and *in vivo* model.

Keywords: Gallic acid, graphene oxide, nanoparticles, toxicity, zebrafish embryo

INTRODUCTION

In recent years, the advancement and development of nanotechnology have been used in various kinds of areas such as engineering, biotechnology and also in medicine. Nanomaterial can be defined as a particle or constituent that is produced by nanotechnology at nano-scale dimension. Nanomaterials characteristic which have small properties in size can aid in the study of drug mechanism to increase the therapeutic efficiency. There is a significant advantage of using nanomaterial in medicine for the diagnosis and treatment of diseases, especially in drug delivery. The ability of nanomaterials is mostly based on their physical properties which can carry drug or compound on their surface plane. Thus, it can enhance the drug to transport directly into the targeted site.

Graphite is a carbon compound made up by single or multi-layer sheets of graphene films consist of single lattice-shaped carbon layer with atomic thickness (Sheshmani & Fashapoyeh, 2013). Meanwhile, graphene oxide (GO) consists of a single-atom-thick layer of graphene sheets with hydroxyl, carboxylic acid and epoxide groups on the surface plane (Dikin et al., 2007). It is produced when graphite undergoes oxidation process, in which oxygenated functionalities are introduced in the graphite structure, and thus expand the layer separation. This structure makes the material more hydrophilic on its basal planes and edges which aid GO to produce water stability suspension and is easily exfoliated into monolayer sheets (Sheshmani & Fashapoyeh, 2013).

GO nanoparticle has tremendous properties and application features according to its physical (light), strength, good thermal, and electrical conductivity such as polymer composites, sensors, 'paper'-like materials, field-effect transistors, energy-related materials and biomedical applications (Yang et al., 2013; Geim & Novoselov., 2007). GO can also be applied in energy storage, nanoelectronics devices, biosensors, drug delivery, cell imaging and tissue engineering (Yang et al., 2011). GO is fluorescent, which makes it especially suitable for various medical applications. Previous studies have reported that GO has anti-microbial activity against gram-negative and gram-positive bacteria (Hu et al., 2010; Santos et al., 2012; Shamsi et al., 2018). In addition, due to its low toxicity, GO has also been investigated for targeted drug delivery in cancer therapy (Chang et al., 2011). GO has a

high surface area and this enables a lot more drugs to be loaded onto its surface. This could potentially reduce the side effects of the current cancer treatment (Danovich et al., 2017).

Gallic acid (GA), 3,4,5-trihydroxybenzoic acid, a type of phenolic acid, is commonly found in gallnuts, sumac, oak bark and many other plants (Dorniani et al., 2012). Previous study has reported that GA has anti-cancer properties by suppressing tumor angiogenesis (Lu et al., 2010). GA also dominates various physiological functions like anti-aging, anti-inflammatory, anti-carcinogenic and anti-melanogenic activity (Dorniani et al., 2016). However, GA suffers a short half-life when administered *in vivo*. GA has specific conformation with three adjacent aromatic phenoxyl groups involved in intra- and inter-molecular hydrogen bonding, which is exhibited in binding and it also exhibits strong chelating abilities with numerous inorganic ligands and proteins (Masoud et al., 2012; Rawel et al., 2006). These unique characteristics allow GA to be modified and loaded to the drug carrier, such as GO.

Zebrafish has emerged as a powerful *in vivo* model system for small molecule screening which is utilized as the alternative model organism for rodent. It is a good model for its unique characteristics which are small, transparent and fast developing eggs that aid high throughput chemical screening and short generation times (Gerlai et al., 2009). In addition, zebrafish also features 70% of genetic homology to human, robust, phenotypes, high-throughput genetic and it also exhibits similar physiological responses, especially during the development of chronic diseases (Howe et al., 2013). Zebrafish can act as an attractive model for studies that aimed at understanding toxic mechanisms, morphological assessment and environmental risk assessment of chemicals such as oxidative stress indices and anti-oxidant parameters.

In this study, we have selected GO as the nanocarrier to create composite nanoparticles that incorporate GA for active drug delivery, GAGO. This nanocompound is believed to produce beneficial synergistic properties especially in anti-cancer (Dorniani et al., 2016) and anti-microbial (Shamsi et al., 2018) activities. However, the underlining toxic effects of GAGO are still largely unclear, especially at the critical period of embryogenesis. Thus, the present study was intended to evaluate the toxicity and teratogenicity effects of a newly formulated nanocomposite compound, as well as its pure forms, GA and GO, in zebrafish embryogenesis.

MATERIALS AND METHODS

Graphene Oxide (GO) Synthesis

An improved Hummers method was used to synthesize GO using graphite powder (Marcano et al., 2010). A 9:1 mixture of concentrated H_2SO_4/H_3PO_4 (360:40 ml) was added to a mixture containing 3 g graphite powder. While stirring, 18 g of $KMnO_4$ was added gradually and slowly to the above reaction mixture, at temperature of 40^{o}C. Then, the mixture was

heated up to 50°C and continued stirring for 12 hr. The reaction mixture was cooled to room temperature for 2 hr and poured onto ice bath (400 ml) with the addition of 3 ml H_2O_2. A centrifugation technique (Sorvall, USA) at 1073 x *g* was used to decant the supernatant from the mixture above. The suspension was washed in succession with 1:1:1 ratio of 200 ml of distilled water, HCl and ethanol to remove any impurities by centrifugation (1073 x *g*, 15 min, room temperature). The suspension obtained was vacuum-filtered using PTFE membrane (50 mm of diameters and 0.45 µm pore size) (Sigma-Aldrich, United States) and left to dry overnight at room temperature.

Preparation of GAGO Nanoformulation

Pure drugs, GA (0.25 g) and GO (0.05 g), were dissolved in 50 ml of distilled water (pH 4.71). The mixture was stirred for 16 hr, centrifuged at 1073 x *g* for 15 min and washed thoroughly in distilled water to remove any unreacted GA. The suspension was stored overnight in an oven at 40°C. Then, the dry suspension were weighted and stored in glass tube (Dorniani et al., 2016).

Characterization of GO and GAGO

The synthesized GO was characterized by using a UHTS 300 Raman spectroscopy (WITec, Germany) with an excitation wavelength of 532 nm. The morphology of GO nanocarrier and GAGO nano-formulation was observed on a scanning electron microscope (SEM) (FEI, USA). Fourier transform infrared spectra (FTIR) (Shimadzu, Tokyo, Japan) was performed on the synthesized GO, GAGO and pure GA using the KBr disc method (Forato et al., 1998). The percentage yield of GAGO was determined for three different batches. The loading capacity and efficiency of GAGO were measured using UV-Vis spectrophotometer (Biorad, USA).

Zebrafish Embryo Toxicity Assessments

The Danio Assay Kit for toxicity assessment was purchased from the Danio Assay Laboratories (Danio Assay Laboratories Sdn. Bhd, UPM, Malaysia), which was equipped with 300 live zebrafish embryos, 96 well plates, 500 ml of Danio-embryo media containing 0.1% DMSO and manual instruction. The wild-type Zebrafish (AB strain) was maintained by Danio Assay Laboratories according to standard in a recirculation system, and under the permission of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia.

The principle of embryonic toxicity assessment on zebrafish embryo was based on the method developed by Schulte and Nagel (Schulte & Nagel, 1994; Nagel, 2002). Briefly, 4 hr post-fertilization (hpf) healthy embryos were transferred into 96-well culture plates (1

embryo in 200 µl of Danio embryo media per well) and acclimatized for 24 hr in a 12-hr light-12-hr dark (LD) cycle. Any dead or unfertilized embryos were removed, and the old medium was replaced with new embryo media. The viable embryos were then treated with different concentrations of GAGO (0-500 µg/ml) at 28°C in a semi-static condition for 96 hr (24 hpf to 120 hpf). GA, GO and distilled water were used as controls. All solutions were refreshed every 24 hr. The different toxicological end-points including mortality rate, heart rate and hatching rate, were observed and captured by using an inverted microscope (Olympus IX73, equipped with DinoCapture camera) at 24 hr, 48 hr, 72 hr and 96 hr post-exposure. The mortality of embryo was indicated when it formed coagulation and absence of heartbeat (Nagel, 2002). Survival rate was analysed by counting the number of live embryos or larvae at each time point. The hatching rate was recorded at 24 hr and 48 hr post- exposure. Dead embryos were removed during the observation time to avoid any contamination. Three independent experiments were performed for each treatment group ($n \geq 10$ embryos per exposure group).

Statistical Analysis

Differences between groups were analysed by Log-rank (Mantel-Cox) test, one- or two-way ANOVA with Tukey's and Dunnet post-test comparison where indicated. All experiments were repeated three times independently. Statistical analyses were performed by using GraphPad Prism 7.0d statistical analysis software (GraphPad Software, La Jolla California USA). Data was presented as mean ± standard error of the mean (SEM). A p value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

The Characterization of GO and GAGO

The GO obtained was observed to be black in colour and appeared flaky (Figure 1A) with a relatively large surface area and morphology that resembles thin folded and crumpled paper under SEM (Figure 1D). The Raman spectra of GO showed significant structural changes and broadening of the D band at 1373 cm^{-1} and 1908 cm^{-1} of G band (Figure 1C). Following the successful synthesis of GO, GA was loaded onto GO, to produce GAGO (Figure 1B), which percentage yield (88.4%), loading of GA (0.382 g/g of GAGO) and loading efficiency of 33.79%. The GAGO obtained was in the form of black thin film (Figure 1B) and SEM image showed the thickness of the GAGO structure, as compared to pure GO (Figure 1E), which indicates the loading of GA.

In order to investigate the different types of interactions, the pure GA and GO as well as the new formulated GAGO were further characterized by using FTIR. This analysis has been performed to investigate the structure and presence of functional groups of the

materials. The presence of new peaks (labelled as black spots) as shown in Figure 2 for GAGO at around 3734 cm^{-1} corresponds to the characteristics peak at 3730 cm^{-1} that appears as well in GA (Figure 2A). Then, the peak in the region 2362 cm^{-1} and 2117 cm^{-1} can also be assigned to ascertain the attachment of GA to the GO nanocarrier. The infrared spectrum of GAGO nanoformulation shows the characteristic peaks of both GO nanocarrier and GA, which suggest the successful loading of GA, hence the formation of GAGO nanoformulation.

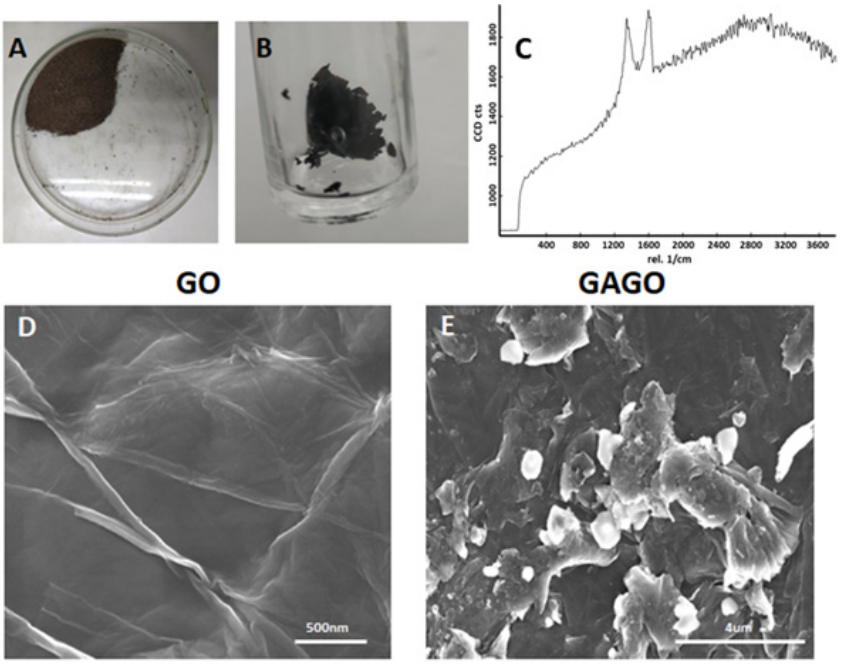


Figure 1. (A) GO flakes synthesized by a modified Hummer's method (B) Freshly prepared GA loaded GO (GAGO). (C) Raman spectra of GO obtained at 532 nm. FESEM images of (D) GO and (E) GAGO at 100x magnification. Scale bar represents 500 nm for GO and 4 µm for GAGO.

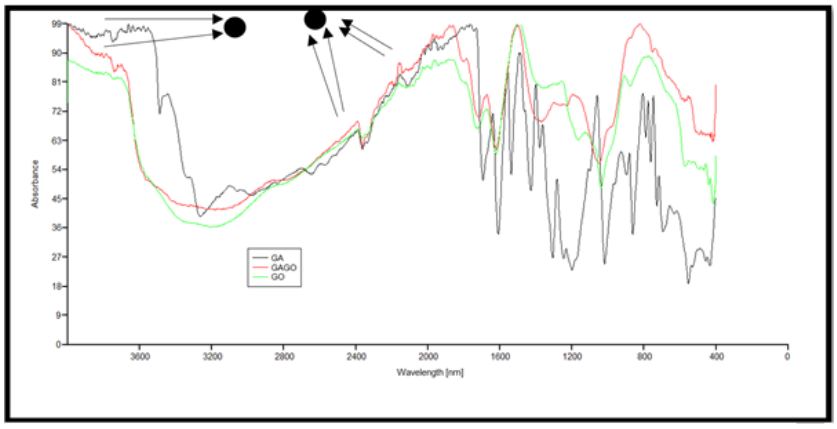


Figure 2. FTIR spectra of (**A**) pure GA (**B**) GAGO and (**C**) GO. The black spots depict new peaks that exist on GAGO spectrum that correspond to the peaks on GA spectrum.

Survival Rate of Zebrafish Embryo Treated with GA, GO and GAGO

In order to assess and compare the possible toxicity effects of GAGO during zebrafish embryogenesis, the survival rate of zebrafish embryos exposed to different concentrations of GAGO (0-500 µg/ml) as observed at 24, 48, 72 and 96 hr post-treatment (Figure 3A-C). In this study, pure GA and GO were used as comparisons to GAGO, concentrations of pure GA (0-191 µg/ml) were based on the percent of drug loading on GO (0-500 µg/ml) obtained during GAGO preparation, and water was used as control. The total percentage of survival rate was represented by dead and unfertilized embryos which exhibited coagulated embryos, lack of somite formations, non-detachment of the tail and absence of heartbeat (Busquet et al., 2013).

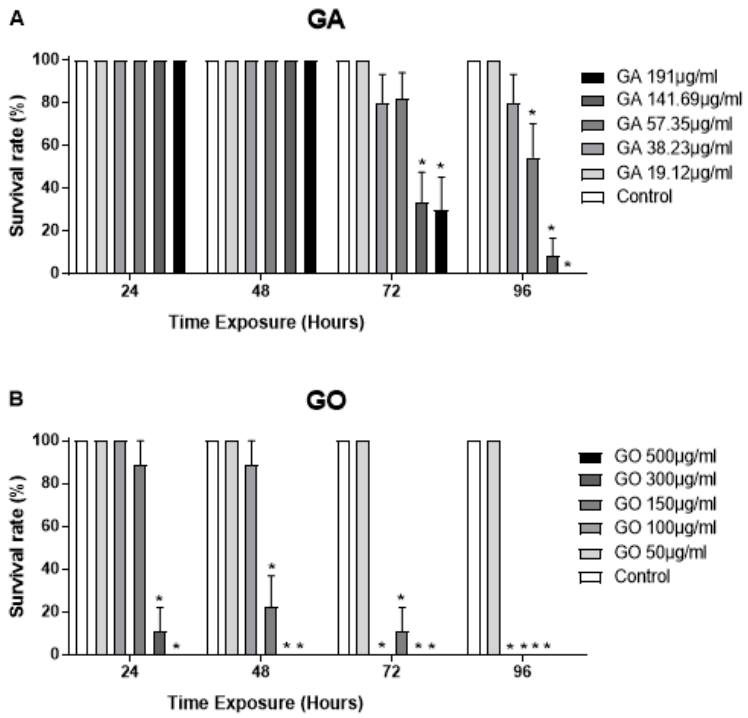
As shown in Figure 3A-B, both pure GA and GO caused significant toxicity to the zebrafish embryos. The two highest concentrations of GA (141.69 and 191 µg/ml) increased the mortality rate of treated embryos, with less than 50% and 10% survival recorded at 72 hr and 96 hr post-treatment, respectively. Compared to GA, pure GO shows more potent toxicity to the embryos, with none survival recorded as early as 24 hr post-treatment at 500 µg/ml (Figure 3B). By 96 hr, complete mortality was recorded in ≥100 µg/ml of GO. However, all embryos treated with the lowest concentration of both, pure GA (19.12 µg/ml) and GO (50 µg/ml), survived this study, and were comparable to the control group. The potential toxicity of GA and GO might be due to their structure that contains multiple hydroxyl groups, particularly in B-rings, which significantly increased the production of reactive oxygen species (ROS) and free radicals that lead to the induction of oxidative stress, results in interruption of normal biological function during the developmental period of zebrafish embryo (Lee & Lee, 2006; Li et al., 2016). This is in contradiction with previous study reported that GA (up to 120 µg/ml) showed no toxicity when exposed to zebrafish embryo for up to 48 hr (Singulani et al., 2017). Together, these data indicate the potential toxicity of prolong exposure of pure GA and GO at high concentrations during zebrafish embryogenesis.

Interestingly, embryos treated with GAGO shows significant improvement in survival rate compared when they were treated with similar concentration of pure GA or GO (Figure 3C). Unlike toxicity seen in GA and GO, treatment with 50-150 µg/ml of GAGO has no significant effect on the survival rate, with 90% survivability of embryos was recorded, at all measured time points, throughout 96 hr exposure. The only fatal concentrations that caused 100% mortality in GAGO were seen at 300 and 500 µg/ml, as early as 24-hour post-treatment. These findings indicate that the toxicity effects of GA and GO, but not GAGO, are based on time- and dose-dependent manners.

The improvement on survival rate observed in GAGO-exposed group might be contributed by the structure of GAGO. Structurally, GO having hydroxyl, epoxy, and carboxyl groups on its surface, which can be covalently bonded with another particle

to functionalize as potential drug treatment (Stankovich et al., 2006). Previous study has reported that the surface charge of graphene has a strong impact on the disruption of red blood cell membranes, which is attributed to the strong electrostatic interactions between negatively charged oxygen groups on the GO surface and positively charged phosphatidylcholine lipids on the cell membranes (Hu & Zhou, 2013). Thus, it is possible that these functional groups may have interacted with the membrane encapsulated the embryos and induces toxicity.

On the other hand, GA naturally works as anti-oxidant, which can inactivate the free radicals reaction hence preventing the building up of oxidative stress. In addition, GA also has phenolic groups that are a source of readily available hydrogen atom, which can delocalize free radicals over its phenolic structure (Nikolic, 2006). Therefore, it is possible that the functionalization of GO, by loading with GA, might has naturalize the negative charges in the GO structure, hence hindering the toxicity effect of pure GO. Moreover, the structure of sharp edge of GO also could potentially disrupt the membrane of embryo that lead and cause death of embryos (Qiu et al., 2018). However, when GO as loaded with GA, the sharp edge surface might be masked and protected from disrupting the membrane of zebrafish embryo, hence increasing the survivability of zebrafish embryo in GAGO treatment group (Sahay et al., 2010).



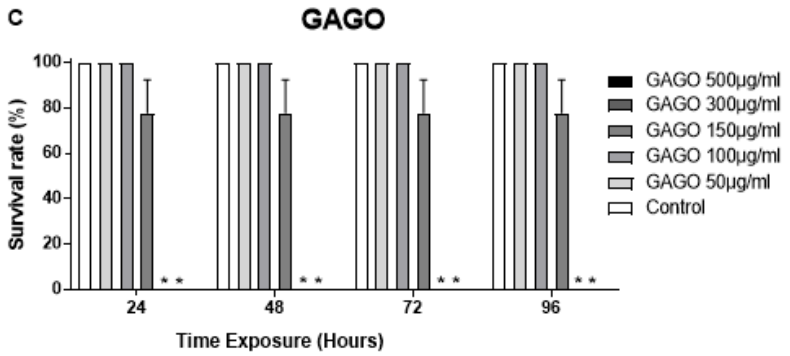


Figure 3. Effects of different treatments on survival of zebrafish (*Danio rerio*) embryos during 96 hr time course. Embryos of zebrafish were exposed at different concentrations of either GA, GO, or GAGO (50, 100, 150, 300, 500 µg/ml). Distilled water was used as control. Data were averaged from three independent experiments and are shown as mean ± SEM. Significant differences between experimental groups are denoted by "*" (One-way ANOVA, $p < 0.05$).

Hatching Rate of Zebrafish Embryo Treated with GA, GO and GAGO

Hatching rate is one of the important parameters to determine the toxicity status of nanomaterials using zebrafish model. Zebrafish embryo normally hatched between 48 and 72 hpf, in which occur at 24 and 48 hr post-treatment in this study, and it is considered as one of the critical stages during embryogenesis (d'Amora et al., 2017). The hatching rates of zebrafish embryos exposed to five different concentrations of GA, GO and GAGO are shown in Figure 4A-C.

In this study, embryos treated with ≤57.35 µg/ml of pure GA showed 100% normal hatching rate (Figure 4A). However, treatment with ≥141.69 µg/ml of GA significantly caused embryonic development delayed, with only 50% hatching rate was recorded between 24 hr and 48 hr post-treatment. As for GO-treated group, less than 20% embryos hatched was recorded when they were treated with ≥100 µg/ml of GO (Figure 4B). The only concentration that did not affect embryos hatching rate in GO was 50 µg/ml, with all embryos within this group hatched between 24 hr and 48 hr of exposure and were comparable to the control group. In contrast to GA- and GO-treated groups, a remarkable improvement in hatching rate was observed in GAGO when they were treated with similar concentration of pure GA (141.69 µg/ml) and pure GO (300 µg/ml) (Figure 4C). In addition, all concentrations except 500 µg/ml, showed normal hatching rate based on the fertilization period of embryos, and they were comparable to the control group. Although Figure 3C recorded that none of the embryos survived at 24 hr post-exposure to 500 µg/ml of GAGO, 60% of these embryos were found dead in larvae state (Figure 4C). Our findings indicate that GA, but not GO and GAGO, exhibited a time- and concentration-dependent behaviour. On the other hand, GO and GAGO have only affected the embryos hatching activity in a dosage dependent manner.

The ability of pure GA to simply dilute and adhere onto the surface of the chorion of zebrafish embryo might partially contribute to the lower hatching rate observed in GA-treated embryos (Ong et al., 2014). Moreover, another study also reported similar finding, in which, graphene is adhered firmly to chorion and induced hatching delayed during zebrafish embryogenesis (Manjunatha et al., 2018; Liu et al., 2014). Adherence of GA and GO might cause clogging in the chorion pores, resulting in interfering of hatching enzyme and oxygen exchange, thus lead to hypoxia in zebrafish embryo (Liu et al., 2014; Yan et al., 2012).

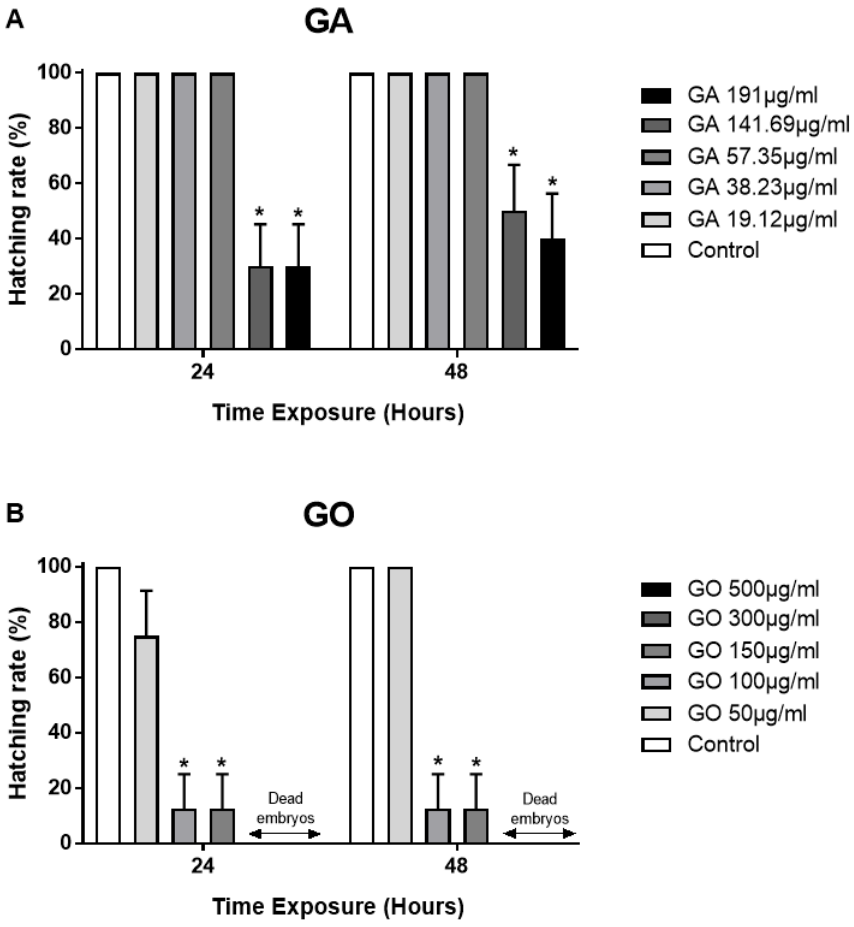


Figure 4. Hatching rate (%) of zebrafish (*Danio rerio*) embryos exposed to different treatments of either GA, GO, or GAGO (50, 100, 150, 300, 500 µg/ml). Distilled water was used as control. Data were expressed as mean ± S.E.M. Significant differences between experimental groups are denoted by "*" (Two-way ANOVA, followed by a post hoc test: Dunnet's, $p < 0.05$).

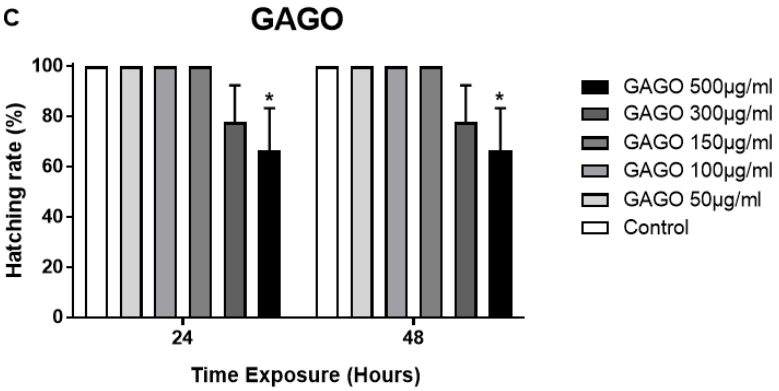


Figure 4. (Continued)

Heart Rate of Zebrafish Embryo Treated with GA, GO and GAGO

Heart is the first organ to develop and function during zebrafish embryonic development (Bakkers, 2011). Heartbeat measurement is important in assessing cardiac function and commonly used to evaluate the toxicity of a toxicant (De Luca et al., 2014). Furthermore, heartbeat induction is also sensitive to nature and can easily be detected due to the embryo characteristic that is transparent and can be visualized at single cell resolution (Verkerk & Remme, 2012). Furthermore, the normal heart rate of zebrafish embryo ranged between 120-180 beats per minute (Baker et al., 1997). The heart rate of embryos exposed to pure GA, GO and GAGO is shown in Figure 5A-L.

As shown in Figure 5, heart rate changes were found in a dose- and time-dependent manner with pure GA and GO exposure, but not GAGO. The heart rate of GA-treated embryos in most concentrations (≤141.69 µg/ml) was within a normal range for up to 48 hr exposure (Figure 5C-D). However, at 72 hr and 96 hr post-treatment, statistically significant decrease was observed at ≥141.69 µg/ml and ≥57.35 µg/ml of GA, respectively. Other GA concentrations show no significant effect on the heart rate and were comparable to the control group. Since all the embryos treated with ≥300 µg/ml of GO were found dead at 24 hr exposure (Figure 3), a remarkable decrease in the heart rate was in consistent with the data (Figure 5E), and it further decreased in all concentrations, except for 50 µg/ml, throughout the 96 hr exposure (Figure 5E-H). GAGO treatment has significantly reduced the heart rate when embryos were treated with ≥150 µg/ml at 24 hr exposure, however, no further decrease was seen and it was maintained throughout 96 hr of exposure. GAGO also posed significant improvement in embryos treated with similar concentrations of GO (Figure 5E-L).

The highly toxicity posed in GO might induce embryonic bradycardia which leads to cardiac arrhythmia (Manjunatha et al., 2018). Bradycardia can be a result of embryonic

distress and/or severe hypoxia. Similarly, this finding was supported by a previous study reported that multi wall carbon nanotube (MWCNT) and GO can alter the heart rate, while reduced graphene oxide (RGO) does not have such effect (Liu et al., 2014). Besides, these results also indicate that combination of GO and GA has promising impact in reducing the toxicity of GO.

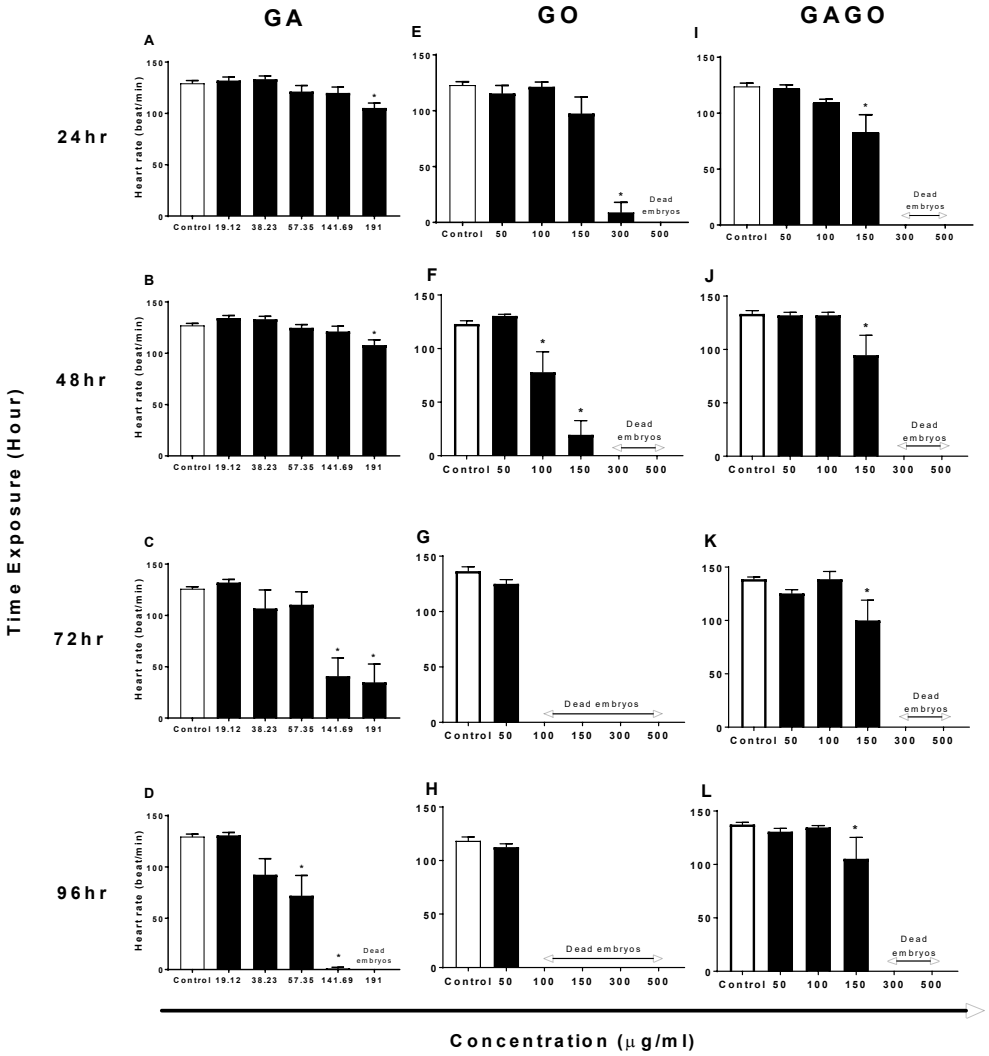


Figure 5. Heart beats of zebrafish embryos in the presence of GA (A-D), GO (E-H) or GAGO (I-L) at different concentrations (50, 100, 150, 300, 500 µg/ml). Embryos treated with GA, GAGO and GO exhibit significant bradycardia at all treated groups. Data were expressed as mean ± S.E.M. Significant differences between experimental groups are denoted by "*" (One-way ANOVA, followed by a post hoc test: Dunnet's, $p < 0.05$).

CONCLUSION

In this study, GA, GO and GAGO were found to induce toxicity based on time- and dose-dependent manner. High mortality, delayed hatching rate and reduced in heartbeat were recorded at higher concentrations of all groups. The present finding shows that the newly-formulated GAGO nanoparticle reduced the toxicity of pure GO and GA during zebrafish embryogenesis, by improving the survival rate, hatching rate and heart rate. The safe concentration of GAGO was recorded between 0-150 μg/ml, in which one-fold higher than pure GA and GO. However, further study is still needed to correlate the toxicity of GAGO with its effective concentration through *in vitro* and *in vivo* studies.

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REFERENCES

Baker, K., Warren, K. S., Yellen, G., & Fishman, M. C. (1997) Defective "pacemaker" current (lh) in a zebrafish mutant with a slow heart rate. *Proceedings of the National Academy of Sciences of the United States of America, 94*(9), 4554-4559.

Bakkers, J. (2011). Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovasc Research, 91*(2), 279-288.

Busquet, F., halder, Braunbeck, T., gourmelon, Lillicrap, A., Kleensang, A., … walter-rohde, S. (2013). OECD guidelines for the testing of chemicals 236 - fish embryo acute toxicity (FET) Test. *The OECD Observer. Organisation for Economic Co-Operation and Development.* Retrieved July 26, 2013, from https://www.oecd-ilibrary.org/environment/test-no-236-fish-embryo-acute-toxicity-fet-test_9789264203709-en

Chang, Y., Yang, S. T., Liu, J. H., Dong, E., Wang, Y., Cao, A., … & Wang, H. (2011). *In vitro* toxicity evaluation of graphene oxide on A549 cells. *Toxicology Letters*, *200*(3), 201-210.

d'Amora, M., Camisasca, A., Lettieri, S., Giordani, S., Amora, M., Camisasca, A., & Giordani, S. (2017). Toxicity assessment of carbon nanomaterials in zebrafish during development. *Nanomaterials*, *7*(12), 414-425.

Danovich, M., Aleiner, I. L., Drummond, N. D., & Fal'ko, V. I. (2017). Fast relaxation of photo-excited carriers in 2-D transition metal dichalcogenides. *IEEE Journal of Selected Topics in Quantum Electronics*, *23*(1), 168-172.

De Luca, E., Zaccaria, G. M., Hadhoud, M., Rizzo, G., Ponzini, R., Morbiducci, U., & Santoro, M. M. (2014). ZebraBeat: a flexible platform for the analysis of the cardiac rate in zebrafish embryos. *Scientific Reports*, *4*, 1-13.

Dikin, D. A., Stankovich, S., Zimney, E. J., Piner, R. D., Dommett, G. H. B., Evmenenko, G., … & Ruoff, R. S. (2007). Preparation and characterization of graphene oxide paper. *Nature*, *448*(7152), 457-460.

Dorniani, D., Hussein, M. Z., Kura, A. U., Fakurazi, S., Shaari, A. H., & Ahmad, Z. (2012). Preparation of Fe_3O_4 magnetic nanoparticles coated with gallic acid for drug delivery. *International Journal of Nanomedicine*, *7*, 5745-5756.

Dorniani, D., Saifullah, B., Barahuie, F., Arulselvan, P., Hussein, M. Z., Fakurazi, S., & Twyman, L. J. (2016). Graphene oxide-gallic acid nanodelivery system for cancer therapy. *Nanoscale Research Letters*, *11*(1), 491-499.

Forato, L. A., Bernardes-filho, R., & Colnago, L. A. (1998). Protein structure in KBr pellets by infrared spectroscopy. *Analytical Biochemistry*, *259*, 136-141.

Geim, A. K., & Novoselov, K. S. (2007). The rise of graphene. *Nature Materials*, *6*(3), 183-191.

Gerlai, R., Fernandes, Y., & Pereira, T. (2009). Zebrafish (*Danio rerio*) responds to the animated image of a predator: towards the development of an automated aversive task. *Behavioural Brain Research*, *201*(2), 318-324.

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., … & Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498-503.

Hu, X., & Zhou, Q. (2013). Health and ecosystem risks of graphene. *Chemical Reviews*, *113*(5), 3815-3835.

Hu, W., Peng, C., Luo, W., Lv, M., Li, X., Li, D., … & Fan, C. (2010). Graphene-based antibacterial paper. *ACS Nano*, *4*(7), 4317-4323.

Lee, K. W., & Lee, H. J. (2006). Biphasic effects of dietary antioxidants on oxidative stress-mediated carcinogenesis. *Mechanisms of Ageing and Development*, *127*(5), 424-431.

Li, X., Gao, A., Wang, Y., Chen, M., Peng, J., Yan, H., … & Chen, D. (2016). Alcohol exposure leads to unrecoverable cardiovascular defects along with edema and motor function changes in developing zebrafish larvae. *Biology Open*, *5*(8), 1128-1133.

Liu, X. T., Mu, X. Y., Wu, X. L., Meng, L. X., Guan, W. B., Ma, Y. Q., … & Li, X. F. (2014). Toxicity of multi-walled carbon nanotubes, graphene oxide, and reduced graphene oxide to zebrafish embryos. *Biomedical and Environmental Sciences*, *27*(9), 676-683.

Lu, Y., Jiang, F., Jiang, H., Wu, K., Zheng, X., Cai, Y., … & To, S. S. (2010). Gallic acid suppresses cell viability, proliferation, invasion and angiogenesis in human glioma cells. *European Journal of Pharmacology*, *641*(2-3), 102-107.

Manjunatha, B., Park, S. H., Kim, K., Kundapur, R. R., & Lee, S. J. (2018). *In vivo* toxicity evaluation of pristine graphene in developing zebrafish (*Danio rerio*) embryos. *Environmental Science and Pollution Research, 25*(13), 12821-12829.

Marcano, D. C., Kosynkin, D. V, Berlin, J. M., Sinitskii, A., Sun, Z., & Slesarev, A. (2010). Improved synthesis of graphene oxide. *ACS Nano*, *4*(8), 4806-4814.

Masoud, M., Hagagg, S., Ali, A., & Nasr, N. (2012). Synthesis and spectroscopic characterization of gallic acid and some of its azo complexes. *Journal of Molecular Structure*, *1014*, 17-25.

Nagel, R. V. (2002). DarT: The embryo test with the zebrafish *Danio rerio*--a general model in ecotoxicology and toxicology. *ALTEX, 19*(1), 38-48.

Nikolic, K. M. (2006). Theoretical study of phenolic antioxidants properties in reaction with oxygen-centered radicals. *Journal of Molecular Structure: THEOCHEM*, *774*(1-3), 95-105.

Ong, K. J., Zhao, X., Thistle, M. E., MacCormack, T. J., Clark, R. J., Ma, G., … & Goss, G. G. (2014). Mechanistic insights into the effect of nanoparticles on zebrafish hatch. *Nanotoxicology*, *8*(3), 295-304.

Qiu, J., Liu, L., Zhu, H., & Liu, X. (2018). Bioactive materials combination types between graphene oxide and substrate affect the antibacterial activity. *Bioactive Materials*, *3*(3), 341-346.

Rawel, H., Frey, S., Meidtner, K., Kroll, J., & Schweigert, F. (2006). Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Molecular Nutrition and Food Research*, *50*(8), 705-713.

Sahay, G., Alakhova, D. Y., & Kabanov, A. V. (2010). Endocytosis of nanomedicines. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *145*(3), 182-195.

Santos, C. M., Mangadlao, J., Ahmed, F., Leon, A., Advincula, R. C., & Rodrigues, D. F. (2012). Graphene nanocomposite for biomedical applications: fabrication, antimicrobial and cytotoxic investigations. *Nanotechnology*, *23*(39), 395101-395110.

Schulte, C., & Nagel, R. (1994). Testing acute toxicity in the embryo of zebrafish, *Brachy danio rerio*, as an alternative to the acute fish test: Preliminary results. *Atla, 22*(1), 12-19.

Shamsi, S., Elias, N., Sarchio, S., & Yasin, F. M. (2018). Gallic acid loaded graphene oxide based nanoformulation (GAGO) as potential anti-bacterial agent against *Staphylococcus aureus*. *Materials Today: Proceedings*, *5*, S160-S165.

Sheshmani, S., & Fashapoyeh, M. (2013). Suitable chemical methods for preparation of graphene oxide, graphene and surface functionalized graphene nanosheets. *Acta Chimica Slovenica*, *60*, 813-825.

Singulani, J. D. L., Scorzoni, L., Gomes, P. C., Nazaré, A. C., Polaquini, C. R., Regasini, L. O., … & Mendes-Giannini, M. J. S. (2017). Activity of gallic acid and its ester derivatives in *Caenorhabditis elegans* and zebrafish (*Danio rerio*) models. *Future Medicinal Chemistry, 9*(16), 1863-1872.

Stankovich, S., Dikin, D. A., Dommett, G. H. B., Kohlhaas, K. M., Zimney, E. J., Stach, E. A., … & Ruoff, R. S. (2006). Graphene-based composite materials. *Nature*, *442*(7100), 282-286.

Verkerk, A. O., & Remme, C. A. (2012). Zebrafish: a novel research tool for cardiac (patho)electrophysiology and ion channel disorders. *Frontiers in Physiology*, *3*, 1-9.

Yan, L., Wang, Y., Xu, X., Zeng, C., Hou, J., Lin, M., … & Liu, Y. (2012). Can graphene oxide cause damage to eyesight? *Chemical Research in Toxicology, 25*(6), 1265-1270.

Yang, K., Feng, L., Shi, X., & Liu, Z. (2013). Nano-graphene in biomedicine: Theranostic applications. *Chemical Society Reviews*, *42*(2), 540-547.

Yang, X., Wang, Y., Huang, X., Ma, Y., Huang, Y., Yang, R., … & Chen, Y. (2011). Multi-functionalized graphene oxide based anticancer drug-carrier with dual-targeting function and pH-sensitivity. *Journal of Material Chemistry*, *21*(10), 3448-3454.