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Dasatinib and PD-L1 inhibitors provoke toxicity and inhibit angiogenesis in the embryo

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ABSTRACT

Dasatinib is a targeted cancer therapy, while programmed death ligand 1 (PD-L1) inhibitors are a form of immune checkpoint therapy used to treat various types of cancers. Several studies showed the potential efficacy of these drugs in the management of triple-negative breast cancer- an aggressive subtype of breast cancer, which can develop during pregnancy. Nevertheless, side effects of Dasatinib (DA) and PD-L1 drugs during pregnancy, especially in the early stages of embryogenesis are not explored yet. The aim of this study is to assess the individual and combined toxicity of DA and PD-L1 inhibitors during the early stages of embryogenesis and to evaluate their effect(s) on angiogenesis using the chorioallantoic membrane (CAM) model of the embryo. Our results show that embryos die at greater rates after exposure to DA and PD-L1 inhibitors as compared to their matched controls. Moreover, treatment with these drugs significantly inhibits angiogenesis of the CAM. To further elucidate key regulator genes of embryotoxicity induced by the actions of PD-L1 and DA, an RT-PCR analysis was performed for seven target genes that regulate cell proliferation, angiogenesis, and survival (ATF3, FOXA2, MAPRE2, RIPK1, INHBA, SERPINA4, and VEGFC). Our data revealed that these genes are significantly deregulated in the brain, heart, and liver tissues of exposed embryos, compared to matched control tissues. Nevertheless, further studies are necessary to evaluate the effects of these anti breast cancer drugs and elucidate their role during pregnancy.

1. Introduction

The *SRC* kinase family of non-receptor tyrosine kinases play a key role in the development, growth, and metastasis of several human cancers including breast [1–4]. Dasatinib (DA), which is commercially known as Sprycel®, is the first FDA-approved SFK/ABL dual inhibitor administered orally to treat several types of cancers as it is shown to block tyrosine kinases including BCR-ABL1 [5]. More specifically, it is designed to treat chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia [6,7]. Moreover, DA showed stronger potency at inhibiting BCR-ABL1 and TKs than imatinib and nilotinib [8–10]. Additionally, it was successful in inhibiting c-KIT, Src, and c-FMS [5,11]. Due to these benefits, preclinical studies suggested that DA could reduce the growth of breast cancer cells;

Abbreviations: ATF3, activating transcription factor-3; Bcl-2, B cell lymphoma-2; CAM, chorioallantoic membrane; DA, dasatinib; FOXA2, forkhead box-A2; FDA, food and drug administration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; INHBA, inhibin beta-A; MAPRE2, microtubule-associated protein RP/EB family member-2; PDL, programmed death ligand; RIPK1, receptor-interacting serine-threonine kinase-1; SERPINA-4, serpin peptidase inhibitor-4; SPSS, statistical package for social sciences; TNBCs, triple negative breast cancers; VEGF-C, vascular endothelial growth factor-C.

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particularly since DA targets Src and deregulates its phosphorylation. These assumptions were later on confirmed due to the promising results of DA treatment against several types of breast cancer, especially triple-negative breast cancers (TNBCs) [12–16]. However, the drug also comes with a number of side effects including cardiac failure, myelosuppression, gastrointestinal bleeding, hypocalcemia, pneumonia, and bone metabolism [5,17].

On the other hand, immune checkpoint inhibitors have shown significant progress in treating several types of cancers via immunotherapy approaches. Recent investigations have revealed that programmed cell death-1 (PD-1), a type-I transmembrane protein receptor, is expressed on the surface of antigen-presenting cells (macrophages and dendritic cells) and that binding of PD-1 with its ligands, PD-L1 and PD-L2, triggers a cascade of downstream signals that suppress T-cell activation [18]. Therefore, overexpression of PD-1 allows tumor cells to evade the immune response by enabling binding of this ligand to its receptor, resulting in the inactivation of cytotoxic T cells by signaling a reduction in cytokines [19,20]. PD-1/PD-L1 have been widely targeted in clinical trials to block their activity and prevent their binding to enable cytotoxic T cells to function properly and identify and kill abnormal cells [21]. In the last few years, anti-programmed death-ligand 1 (PD-1/PD-L1) agents have been found to play a role in blocking breast cancer, especially TNBC, when administered as a monotherapy or in combination with conventional treatments [22]. Some of the side effects associated with the usage of PD-L1 inhibitors have been summarized in previous studies and reviews; they range from hematological to neurological symptoms that affect various organs [23].

Most of the studies that investigated the efficacy and side effects of DA and PD-1/PD-L1-inhibitor in treating TNBC, were conducted on non-pregnant patients. Nevertheless, as TNBC is one of the most aggressive subtypes of breast cancer that can develope during pregnancy, side effects of these drugs on embryogenesis should be addressed. To date, there have been limited studies that investigated the effects of DA and PD-1/PD-L1-inhibitor separately on embryogenesis [24–26]. However, to the best of our knowledge, there are no studies on their combined effect on the early stages of normal vertebrate development. Thus, we herein explored for the first time, the solo and combined effects of DA, PDL-inhibitors on the early stages of normal development and angiogenesis using avian embryos and its chorioallantoic membrane (CAM) as a model.

2. Materials and methods

2.1. Preparation of inhibitors

DA (AbcamID#: ab142050, Abcam, Cambridge, UK) and BMS-202 (PD-1/PD-L1 inhibitor 2) (AbcamID#: ab231311, Abcam, Cambridge, UK) small molecule inhibitors (25 mg) were prepared by dissolving DA and PD-L in DMSO to achieve a final concentration of 5 μg/mL.

2.2. Evaluation of the effects of DA and PD-L treatment on embryos

Fertilized chicken embryos of the White Leghorn type were bought from the Arab Qatari Company for Poultry Production and incubated at 37 °C with 60 % humidity in a MultiQuip. All procedures were ethically approved by the Institutional Bio-safety committee of Qatar University. Four sets of experiments were carried out, with 35 embryos used for each set of experiments. Each embryo was exposed on day three of incubation with 5, 5 and 5 + 5 μg/mL of DA, PDL1 and DA + PDL1, respectively.

Treatment was performed briefly as follows: a small circular incision was made on the top of the eggshell, and the membrane was carefully removed by adding 100 μl of 1X PBS (Sigma-Aldrich, UK). The respective treatment was added on circular coverslips (Sigma-Aldrich, UK) and placed directly on the embryos. Embryos treated with a 2 μg/mL DMSO were used as control. The eggs were then sealed and incubated for a period of five days, with mortality incidences recorded daily. The embryos were sacrificed on day five and day eight of incubation and their brains, hearts and liver tissues autopsied for macroscopic observation and RNA extraction for RT-PCR analysis.

2.3. Angiogenesis assay

Treated chicken embryos were analyzed on day five of incubation to evaluate the outcome of various treatments on vascular development on the CAM. The CAM of the embryos were treated with the prepared drug (DA, PDL1 and DA + PDL1), which were placed on a circular glass cover slip for 24 and 48 h, respectively, as previously described by our group [27,28]. DMSO-treated embryos were used as controls. After 24 and 48 h of treatment, the vascular development of the CAM was examined daily over a period of three days under a stereomicroscope. Images were captured and the number of branching points and length of blood vessels were quantified using the AngioTool Software 0.6a [29]. Briefly, images were extracted to this program. All extracted images have the same size and magnification with unified AngioTool inputs. As shown in Fig. 1, the blue dots and yellow lines represent the actual blood vessels in the image.

Parameters including vessel diameter and intensity thresholds were set at 10 and 255, vessel thickness at 4 and 5, removed small particles at 200 and filled holes at 150 were set and analysis was run. An excel sheet was generated with different parameters (total blood vessels length and total number of junctions) and values were plotted using GraphPad Prism (version 8.4.3). Three independent sets of experiments were performed to obtain reproducible results.

2.4. RNA isolation and reverse transcription RT-PCR analysis

Total RNA was isolated and purified from brain, heart, and liver tissues of exposed chicken embryos and their respective controls on day 8 of incubation using the AllPrep DNA/RNA FFPE Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA samples were used at a final concentration of 50 ng/μl. Synthesis of the cDNA and PCR amplification were performed using Invitrogen SuperScript™III One-Step RT-PCR System with Platinum™Taq DNA Polymerase (ThermoFisher Scientific, USA), according to the manufacturer's protocol.

RT-PCR amplification was performed using primer sets for the following genes: Activating transcription factor-3 (ATF3), forkhead box-A2 (FOXA2), inhibin beta-A (INHIBA), microtubule-associated protein RP/EB family member-2 (MAPRE-2), receptor-interacting serine-threonine kinase-1 (RIPK-1), serpin peptidase inhibitor-4 (SERPINA-4), vascular endothelial growth factor-C (VEGFC), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH), as previously described [27]. GAPDH was used as an internal control. In each experiment, the positive control for all genes included brain or liver tissue from healthy chicken embryos.

To obtain the relative gene expression, quantification was performed by analyzing RT-PCR images using the ImageJ software 1.52k [30]. The relative expression of these genes in each tissue (the heart, liver, and brain) was calculated based on their intensity, relative to the GAPDH bands. Briefly, each band was outlined individually and the signal intensities in pixels in the area occupied by the band were quantified using the gel analysis tool in ImageJ menu. As a control, this was also repeated for an equivalent area of background for each RT-PCR gel image. The mean pixel intensity of the background was subtracted from the mean intensity of each band. The band intensity for each gene (ATF3, FOXA2, INHBA, MAPRE2, RIPK1, SERPINA4 and VEGFC) after background subtraction was then normalized with the band intensity of GAPDH (after background subtraction). The obtained values were then used to plot the bar graphs using GraphPad Prism.

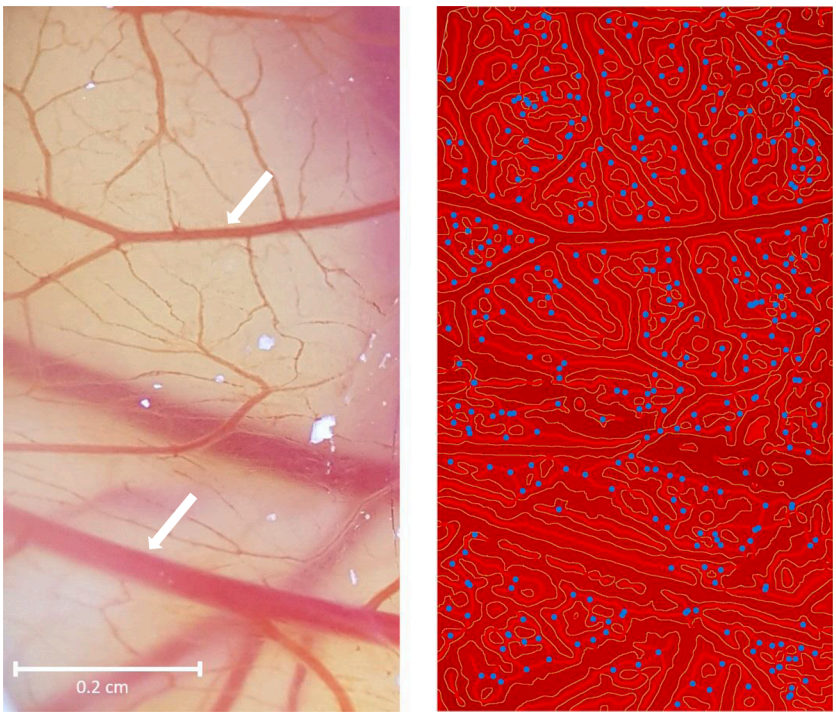

Fig. 1. The image on the left is the captured image of the chicken embryo with blood vessels (white arrows). The image on the right is the derived image in the AngioTool software. The blue dots and yellow lines represent blood vessel junctions.

2.5. Statistical analysis

The statistical significance was performed for each of the three sets of experiments. GraphPad Prism version 8.4.3 was used to estimate the statistical differences between treatment groups. Data were presented as mean ± SEM (standard error of the mean). A Kaplan-Meier Estimator was used to plot the survival curves of treated and control groups, and a log-rank test to detect the significance between the two groups. An independent samples *t*-test was used to calculate the significance between the treatment and control, while a student's *t*-test was used to analyze the blood vessel parameters of treated and control groups. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. The effects of anti-cancer drug treatments on embryogenesis

As described in the methodology section, this study investigated the effects of DA and PD-1/PD-L1 inhibitors on the early stages of embryogenesis using the chicken embryo as a model. One-hundred and thirty four (134) embryos were exposed to DA and PD-1/PD-L1 alone or in combination, of which 35 embryos were treated with a 2 % DMSO as control. Embryos were examined every day for the following five days. The embryos were treated with 5 μg/embryo of each inhibitor alone and 5:5 μg from DA and PD-1/PD-L1 in combination. After two days of treatment (day 5 of incubation), we observed that 33 (53 %) of the 62 embryos died after DA treatment, seven (18 %) of 39 embryos died after PDL treatment, and 20 (~61 %) of 30 embryos died when exposed to the combined treatment of (DA + PDL). Four embryos out of 35 controls (~11 %) died in the same period. While, after 5 days of treatment (day 8 of incubation), only two (5%) of the 39 PDL1- treated embryos died ($p < 0.0001$) in comparison to the control, for which no mortality was recorded (Table 1).

Thus, it is clear that DA and PD-1/PD-L1 inhibitors reduce survival probability significantly, compared to the controls ($p < 0.0001$; Fig. 2). Embryos exposed to DA, and DA + PDL1 inhibitors were euthanized on day 5 and day 8 of incubation and autopsied to isolate the brain, heart, and liver tissues for further investigation.

Table 1
Effect of DA and PD-1/PD-L1 inhibitors on embryos.

Groups of embryos	Sample size	Mortality rate on day 2 after exposure (%)	Mortality rate on day 5 after exposure (%)
DA-exposed embryos	62	33 (53 %)	62 (100 %)
PDL1-exposed embryos	39	7 (18 %)	9 (23 %)
DA + PDL-exposed embryos	33	20 (61 %)	33 (100 %)
Controls	35	4 (11 %)	4 (11 %)

3.2. The impact of DA and PD-1/PD-L1 inhibitors on angiogenesis of the CAM model

The outcomes of the DA, PDL1, and DA + PDL1 were explored on blood vessel development (angiogenesis) using the CAM model of the chicken embryo at day 5 of incubation, as described in the methodology section. The effect of each of the treatments on the total number of junctions and total vessel length was measured (Fig. 3). After 24 h, the anti-cancer drugs slightly reduced the formation of new blood vessels in CAM. While, after 48 h, DA and PDL1 inhibitors significantly reduced the formation of new blood vessels in the CAM, compared to DMSO-treated controls (Fig. 3A).

After 24 h of treatment, the anti-cancer drugs had no significant effect on either the total length of blood vessels or the number of junctions. Embryos exposed to DA, PDL1, and DA + PDL1 treatments did not exhibit significant lower values for the total length of blood vessels, compared to DMSO-exposed controls, with 90, 81 %, and 86 % reductions, respectively ($p > 0.05$) (*Data not shown*). Additionally, these embryos did not show a significant decrease in blood vessel junctions, compared to DMSO-treated controls with decrease of 78 %, 72 %, and 84

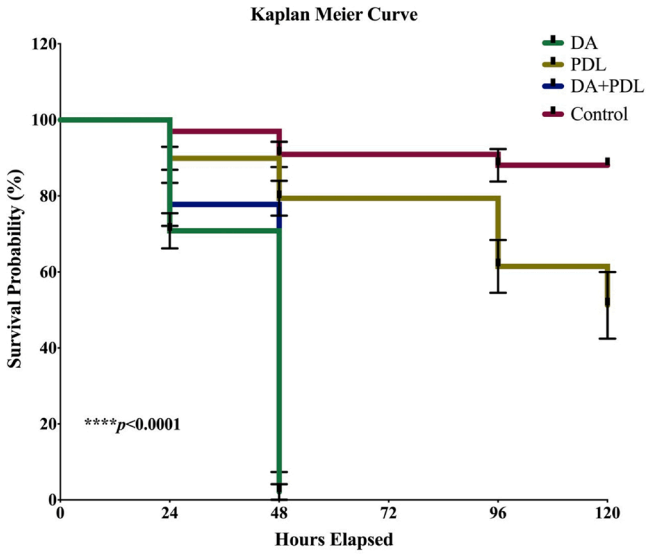

Fig. 2. Kaplan Meier survival curve of treated embryos and their matched controls. Treatment with DA, PDL inhibitor and combination of DA and PDL significantly reduce the survival rate of exposed embryos compared to their control (****$p < 0.0001$).

%, respectively ($p > 0.05$) (*Data not shown*).

However, after 48 h, embryos exposed to DA, PDL1, and DA + PDL1 treatments have significantly lower values for the total length of blood vessels, compared to DMSO-exposed controls with 23 %, 19 %, and 29 % reductions, respectively, ($p = 0.0212$, $p = 0.0315$, and $p = 0.0042$, respectively) (Fig. 3B). Additionally, after 48 h, exposed embryos with DA, PDL, and DA + PDL showed a significant decrease in blood vessel junctions, compared to DMSO-treated controls with reductions of 41 %, 39 %, and 46 %, respectively ($p = 0.0211$, $p = 0.0132$, and $p = 0.0093$, respectively) (Fig. 3C). This analysis demonstrates that DA and PDL1 inhibitors significantly hamper the angiogenesis of the CAM.

3.3. Effects of DA and PD-1/PD-L1 inhibitors on gene expression in different tissues from exposed chicken embryos

RT-PCR analysis was performed to examine the expression of a set of genes related to cell proliferation, angiogenesis, and survival in brain, heart, and liver tissues from both treated embryos and matched controls. The selected panel of genes included ATF3, FOXA-2, INHIBA, MAPRE2, RIPK-1, SERPINA-4, and VEGF-C, which were chosen based on their role in embryogenesis and our previous studies [27,28,31].

Our results showed that in exposed embryo tissues, ATF3, FOXA2, INHBA, MAPRE2, and RIPK1 are significantly overexpressed, while SERPINA-4 and VEGF-C are significantly down-regulated, compared to their matched control tissues (Fig. 4). This pattern was observed in all brain, heart, and liver tissues exposed to each treatment.

4. Discussion

We herein investigate for the first time the outcome of DA and PDL1 inhibitor, which are largely used for the treatment of several types of

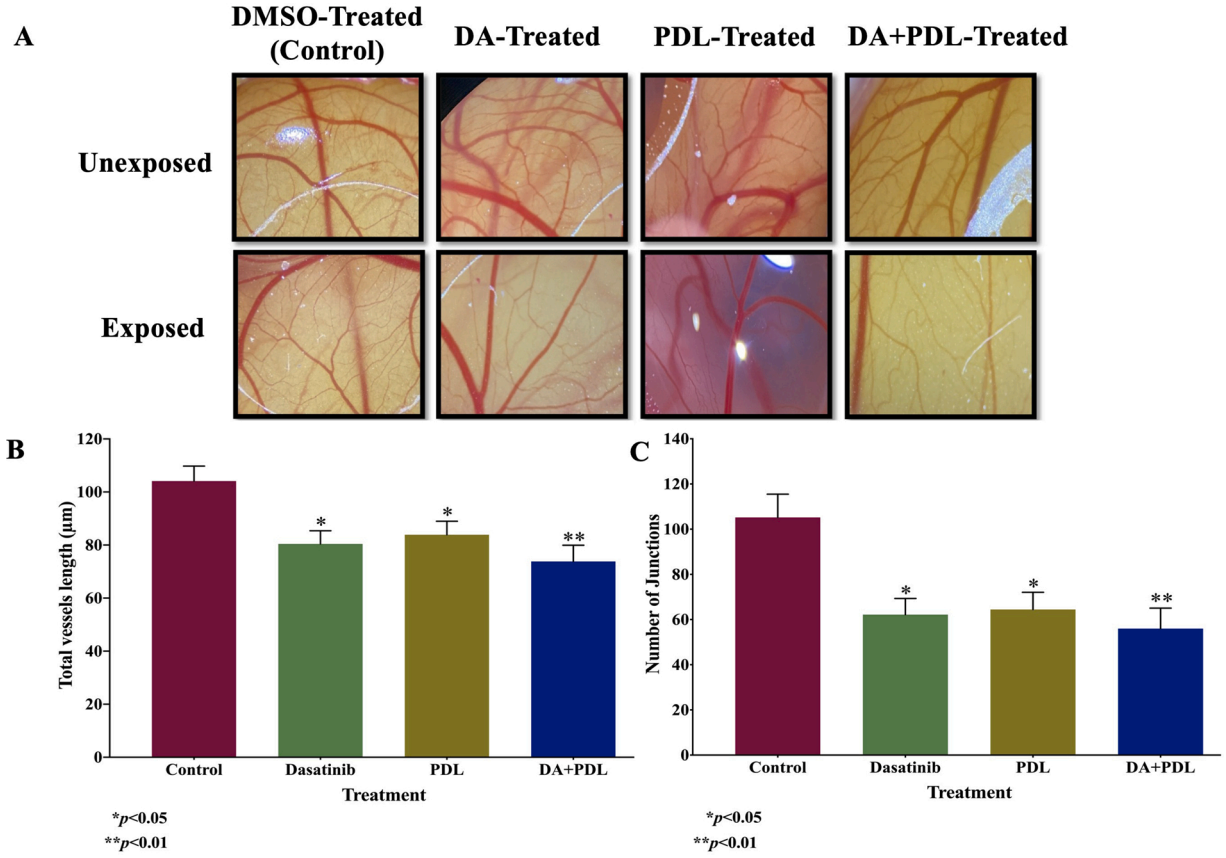

Fig. 3. (A-C). The effects of anti-cancer drugs on angiogenesis of the CAM model 48 h after treatment. This analysis was performed using CAM treated with DMSO (control) and those exposed to DA, PDL inhibitor, and a combination of DA and PDL. (A). The images on the top show the unexposed areas and those on the bottom show areas that were exposed to the treatment (under the coverslip) of the same embryo. Two areas within each individual embryo in both groups were compared to examine total blood vessels length and the number of junctions (B). The total blood vessels length of controls v/s treated embryos (*$p < 0.05$, **$p < 0.01$). (C). The number of junctions of controls v/s treated embryos (*$p < 0.05$, **$p < 0.01$).

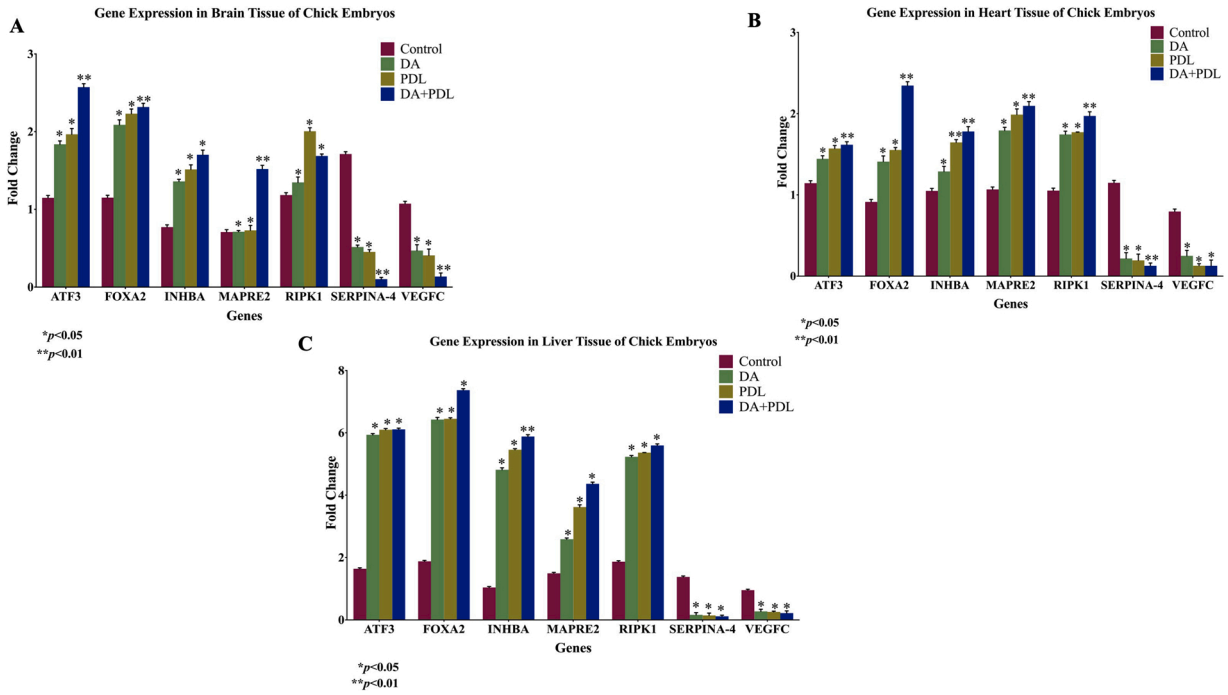

Fig. 4. (A-C). RT-PCR analysis of seven genes (ATF3, FOXA2, INHBA, MAPRE2, RIPK1, SERPINA4, and VEGFC) in brain, heart, and liver tissues of chicken embryos. This analysis was performed in parallel using organ tissues derived from both normal embryos and those exposed to DA, PDL inhibitor, and a combination of DA and PDL. The GAPDH gene was used as an internal control. Quantification data of ATF3, INHIBA, FOXA2, SERPINA4, VEGFC, RIPK1, and MAPRE2 gene expressions in brain (A), heart (B), and liver tissues (C) of embryos treated with anti-cancer drugs (DA, PDL, and DA + PDL) and their matched controls. We note that the ATF3, INHIBA, FOXA2, RIPK1, and MAPRE2 genes are up-regulated, while, SERPINA4 and VEGFC are down-regulated in the tissues obtained from treated embryos, compared to their matched control tissues.

cancers, on the early stages of embryonic development. Although DA and PD-1/PD-L1 inhibitors play a key role in targeted therapy and immunotherapy respectively [6,7,21], there have been reports of their toxicity [32,33]. The toxic effects of DA as a single agent as well as in combination have been observed in preclinical studies (phases I and II) of patients with solid tumors; common side effects include myelosuppression, hemorrhages, musculoskeletal pain, fatigue, anemia, leukopenia, neutropenia, and thrombocytopenia [5,17,34–37]. PD-1/PD-L1 inhibitors can lead to immune-related adverse events [38]; common side effects include diarrhea and nausea, while severe (grades three or four) side effects include damage to the endocrine, gastrointestinal, and respiratory systems [39,40]. Side effects are frequently managed with courses of corticosteroid therapy, but in some severe cases the termination of immunotherapy is suggested [41]. These adverse effects can vary, depending on whether the drug is taken as a single therapy or in combination with other drugs. To the best of our knowledge, no study has reported the potential toxicity of the combination of DA and PDL during embryogenesis. Therefore, in the present work we used the chicken embryo model to explore the toxic outcome of these inhibitors on the early stage of the embryo, while the CAM model was used to investigate their effect on angiogenesis [42,43]. This study demonstrated that treatment with DA or PDL1, whether as single agents or in combination, provoke significant toxicity during the early stage of embryogenesis; while, the combination of DA and PDL1 has a comparatively higher toxic effect with a lower chance of survival when compared to individual treatment. DA and PDL1 also inhibit the formation of blood vessels in the CAM within 48 h of treatment through the impairment of several key genes regulating angiogenesis, proliferation, survival, and apoptosis. We analyzed the expression patterns of ATF3, FOXA2, INHBA, MAPRE2, RIPK2, SERPINA-4, and VEGF-C genes in the brain, heart, and liver tissues of treated embryos and their matched controls. Our data show that ATF3, FOXA2, INHBA, MAPRE2, and RIPK-1 are upregulated while SERPINA4 and VEGFC are downregulated in the brain, heart, and liver tissues of treated embryos, compared with their matched controls, indicating the possibility of major changes on both molecular and physiological levels due to exposure to DA, PDL1, or both. Genes that are overexpressed play a vital role in regulating cell cycle and proliferation in normal conditions, while their overexpression leads to uncontrolled cell proliferation [27,44,45]. ATF3 overexpression in embryos transforms primary cells [46] and enhances the expression of FOXA2, which is associated with proliferation, invasion, metastasis, and epithelial-mesenchymal transition (EMT) [47], that are well known hallmarks of carcinogenesis and embryogenesis. Similarly, INHBA, MAPRE2, and RIPK-1 also regulate cell proliferation and tumor invasion; which are associated with uncontrolled cell growth [45,48,49]. During embryogenesis, INHBA is involved in axis development and organogenesis [50]; a higher expression has been linked to toxic effects. On the other hand, SERPINA4, a down-regulated gene in our exposed embryos, is responsible for various signaling pathways that are essential for several biological functions. Studies have shown that enhanced SERPINA4 levels reduce angiogenesis and inflammation and defend against vascular and organ injury and tumor progression; while, reduced SERPINA-4 expression is associated with septic shock, hypertension, cardiovascular and renal injury, and hepatic neoplasia in animal models [51–53]. These drugs have been shown to inhibit angiogenesis upon exposure; which is confirmed by the down-regulation of VEGF-C, the gene responsible for blood and lymphatic vessel formation [54], indicating that it plays a key role in embryonic development. Studies of mice and zebrafish models have indicated that the down-regulation of VEGF-C results in their accelerated death [55–57]. These results are consistent with observations in the present study in addition to our previous studies in which we demonstrated the toxic effects of nanoparticles and carbon nanofibers on embryogenesis [27,28,31]. In summary, our study has shown for the first time that DA and PD-L1 have a severe toxic effect, both individually and in combination, on the early stage of embryogenesis through the deregulation of essential controller

genes (ATF3, FOXA2, MAPRE-2, INHIBA, RIPK-1 SERPINA-4, and VEGFC) involved in regulating survival, apoptosis, cell proliferation, mitosis, organogenesis, and angiogenesis.

5. Conclusion

Findings of this study demonstrate -for the first time- that exposure to DA and PD-L1 inhibitors has significant adverse effects on the early onset of the normal development of embryos. For the studied concentrations, DA and PD-L1 caused significant mortality in embryos, both individually and in combination, and inhibited angiogenesis of the CAM. Our data suggest that PD-L1 might reduce the toxicity of Dasatinib. Another finding is that the mechanism of toxicity is mainly due to the dysregulation of key controller genes responsible of vital biological events during embryogenesis. However, further *in-vitro* and *in-vivo* studies are needed to confirm our findings using different models of embryogenesis, and to understand their underlying mechanisms. Based on the findings of the current study, the use of these inhibitors during the early stages of pregnancy should be weighed critically for their benefits and risks.

Contribution

Maha Al-Asmakh: conceptualization, writing, supervision, data analysis, provision of resources, project administration, acquisition of funding. Hiba Bawadi: conceptualization and writing. Munia Hamdan: writing and performance of experiments. Ishita Gupta: writing and data analysis. Hadeel Kheraldine and Ayesha Jabeen both contributed and assisted in the experimental techniques with angiogenesis and RT-PCR. Balsam Rizeq: data analysis. Ala-Eddin Al Moustafa: conceptualization, writing, supervision, project administration, acquisition of funding.

Declaration of Competing Interest

"The authors declare no conflict of interest." "The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

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