Hypoxia induced angiogenesis and upregulation of VEGF: An in vivo study using Zebrafish model

Vivek Sagayaraj. R, Navina Paneerselvan, Sankar Jagadeeshan and Raghunathan Malathi

Abstract: Cobalt chloride, a known hypoxic agent is able to induce angiogenesis in zebrafish embryos. The concept of this study is to sense the effect of $CoCl_2$ during development, using zebrafish as model system. Embryos exposed transdermally to varying concentrations (0.5-5µM) of $CoCl_2$ has shown to enhance the formation of blood vessel and intersegmental vessel (ISV), as detected by RBC and alkaline phosphatase staining respectively. Length, size, sprouting junction of blood vessels were measured using the software angioquant and shown significant change with increasing concentrations. An increase in VEGF, VEGF-R2 and HIF-1α mRNA expression were observed through qPCR, while western blotting analysis revealed a considerable increase in VEGF expression and a complete inhibition of VEGF under hypoxia with SU5416, an inhibitor of VEGF signalling. The ability of $CoCl_2$ to induce angiogenesis in zebrafish embryos might be through the enhanced expression of HIF-1α, VEGF, and VEGF-R2, the key player of angiogenesis and the results obtained with SU 5416 is suggestive the involvement of VEGF signalling during embryonic development.

Key Words: Angiogenesis, development, zebrafish, hypoxia, cobalt chloride, VEGF, HIF-1α.

1. INTRODUCTION

Angiogenesis is a highly complex process that plays an intimate role during embryogenesis, vascular development, differentiation, wound healing and organ regeneration [1]. Under hypoxic condition, angiogenesis is shown to get up-regulated with increased in HIF-1α expression and other common set of angiogenic factors that are involved in cell growth, migration, glucose metabolism etc. [1,2]. These include vascular endothelial growth factor (VEGF) and its receptors, including VEGFR-1 (also called *flt-1*) and VEGFR-2 the major regulator of angiogenesis promoting endothelial cell differentiation, survival and migration [2,3] largely through stabilization of hypoxia inducible transcription factors (HIFs).

Zebrafish is used as an alternative vertebrate model to study angiogenesis as it possess a complex circulatory system comparable to mammals with reasonable counterparts and evaluation of blood flow is extremely easy to score in zebrafish embryos, making it an ideal model for the study of angiogenesis [4]. The significant similarity in protein sequences, conservation of developmental processes leading to organogenesis, and common appearance of pathophysiologic mechanisms all contribute to pull out zebrafish in biomedical research [5].

Broad research is carried out under hypoxia with metals and chemicals, yet the mechanism is still under debate. Only few metals such as mercury, copper, nickel, lead, and cobalt were evaluated for their effects on hatching and survival in zebrafish embryos [6]. Reports suggest that cobalt chloride, nickel chloride and desferrioxamine [7] can act as hypoxic mimicking agents exhibiting angiogenic activity. Cobalt chloride, a known chemical inducer is known to evoke hypoxic like responses. Cobalt chloride has gained much importance in tail fin regeneration, heart regeneration of zebrafish and regenerative angiogenesis study [1,6]. With its biosignificance in regenerative angiogenesis, the effect of cobalt chloride on developmental angiogenesis is presently evaluated using zebrafish as a model organism and attempts have been made to study the affiance of $CoCl_2$ during embryogenesis.

In the present study, we examined the expression of vascular endothelial growth factor A and hypoxia inducible factor-1 under hypoxic

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condition created due to exposure of $CoCl_2$ and its dependence on the VEGF pathway in zebrafish embryos. It is found that $CoCl_2$ in the range of 0.5-2μM can persuade angiogenesis by promoting formation of ISV and its vasculature with stabilized HIF-1α resulting in increased VEGF expression. The results obtained were assessed with that of SU5416 a potential inhibitor of VEGF signaling pathway.Comparing the results obtained with $CoCl_2$ to that of SU5416 suggests that VEGF *via* HIF 1α signalling might play a pivotal role during critical events of embryonic development.

2. Materials and methods

2.1 Plastic wares, glass wares:

Fish tanks, petri plates, pasteur pipettes, glass slides, were obtained from local sources.

2.2 Chemicals and reagents

$CoCl_2$, o-dianosidine, NBT, BCIP, SU 5416 were purchased from Sigma Aldrich Chemicals. Pvt. Ltd. (USA).Tri Reagent was purchased from Invitrogen (USA) and all other chemicals used were of molecular grade. Cobalt chloride was dissolved in molecular grade water at stock concentrations of 10 mM and then diluted to 0.5, 1 and 2μM. 1μM SU 5416 was used as a positive control. Control embryos were treated with embryo medium. VEGF and β-actin antibody were purchased from R&D systems and secondary antibodies were from Santa Cruz Biotechnology.

2.3 Zebrafish maintenance:

Adult zebrafish (wild type) were purchased from local aquarist brought up as mentioned in ''***The Zebrafish handbook: a laboratory use of Zebrafish***'', kept in fish tanks for experimental study

2.5 Morphometry analysis:

Zebrafish embryos treated with $CoCl_2$ transdermally were maintained at 28º C, for further analysis.

Morphological features were documented using light microscope (Euromax, Noveland), at 4X resolution and blood vessel length, size; sprouting junctions were measured using Angioquant software (Version 1.33, MathWorks)[8].

2.6 Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from treated zebrafish embryos using Tri Reagent (Invitrogen). RNA was quantified using nano drop (Thermo Scientific) and reverse transcribed using random primers (Promega), DNTPs (Invitrogen), reverse transcriptase (MMuLV), reverse transcriptase buffer, riboblock (Thermo Scientific), Molecular grade water (Sigma) in a PCR vial incubated at 42ºC to perform reverse transcription.

2.7Analysis of mRNA expression by Real Time Polymerase Chain Reaction

RT-PCR was performed in Biorad PCR system (USA). Reaction was carried using Sybr green master mix (Kapa), Template, primers, and molecular grade water. The reaction mixture contains 5μl Sybr green Master mix, 1μl forward primer and 1μl reverse primer, 0.5μl cDNA template, 2.5μl molecular grade water. Real- time Polymerase chain reaction was performed for VEGF-A, VEGF-R2, HIF-1α and β-Actin serving as internal control with following primer sequences (Table 1).

2.8 Western Blotting Analysis

Protein was isolated from embryos treated with cobalt chloride, quantified using the Lowry's method (9). 50 μg protein from each sample resolved on 10% SDS-PAGE, subsequently transferred to PVDF membranes. Membranes were blocked in 5% milk solution, incubated with primary antibody (R&D systems) in 1:1000 dilution at 4˚C overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilutions) incubation for 1 hour at room temperature. The immunoreactivity was detected by chemiluminescence.

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2.9 RBC Staining

RBC staining was performed from previously described methods [10]. On 72 hpf, embryos were fixed in 4% paraformaldehyde for 30 min and washed with PBS for 3-4 times. Embryos were stained for 30 min in the dark in o-dianisidine 0.6mg/ml, 0.01 M sodium acetate (pH 4.5), 0.65% hydrogen peroxide, and 40% (v/v) ethanol. After 30 min it was washed with PBS for 3-4 times and examined under the microscope and micro photographed.

2.10 ALP Staining

Alkaline phosphatase staining was used to study ISV formation as mentioned [11]. 72 hpf embryos washed with PBS for 3-4 times and fixed in 4% paraformaldehyde (pH – 7.5) for 2 hours at room temperature. Embryos were washed 3-4 times in PBS. It is dehydrated by immersing in 25 %, 50%, 75% and 100% methanol each with 5 min in PBST. For staining embryos were equilibrated in NTMT buffer thrice each with 15 min duration (0.1M Tris-HCl; pH 9.5; 50mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween-20) at room temperature. Once the embryos were equilibrated in NTMT, 4.5μL of 75 mg/mL NBT and 3.5μL of 50 mg/mL BCIP was added. After staining for 20 min, the reaction was stopped by adding PBST. Embryos were then immersed in a solution of 5% formamide and 10% hydrogen peroxide in PBS for 20 min which removed endogenous melanin in the pigment cells and allowed full visualization of stained vessels. It is then examined by compound microscope and photographed.

Table 1: List of primers used in this study.

S.No	Gene	Forward primer	Reverse primer
1	VEGF–A	5′ CTCCTCCATCTGTCTGCTGTAAAG 3′	5′CTCTCTGAGCAAGGCTCACAG 3′,
2	VEGF–R2	5'GGTGAAGAAGGACGATGAGG 3′	5′ ACAGGAATGTTCCTGCTGCT 3′
3	HIF-1α	5′ CTACAATGATGTCATGCTGCC 3′	5′ACACAGAGTGAGTGGCAGAA 3′
4	β-actin	5′TTCACCACCACAGCCGAAAGA3′	5′TCACCACCACAGCCGAAAGA3′

2.11 Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Tukey's Multiple Comparison tests, for comparison between treated and control values using Graph Pad Prism software (Version 5, USA). P values < 0.05, were considered to be statistically significant.

3. RESULTS

3.1 Morphological screening of $CoCl_2$ on zebrafish embryos.

Zebrafish embryos treated with $CoCl_2$ at 0.5, 1 and 2 μM were maintained at 28ºC in six well plates after drug treatment until 72 hpf, to create hypoxic condition. Response to $CoCl_2$ induced hypoxia, zebrafish embryos exhibited enhanced hatching efficiency (Fig.1A), increased heart beat rate (Fig.1B) in parallel with amplified amount of blood vessels with increasing concentrations up to 2 μM.

Angiogenesis involves sprouting of blood vessel. Blood vessel formation and development is very essential during the development stages of the embryo. Angiogenesis is determined in three different aspects *viz.*, length, size and sprouting of blood vessel using angioquant software at 72hpf. Increased blood vessel formation exposing to different doses of $CoCl_2$ is documented using light microscope. The control embryo developed normally at 72hpf, while blood vessel formation was significantly enriched on exposure to $CoCl_2$ with a maximum fold increase of

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2.1 in length, 2.5 in thickness and 2.5 in blood vessel sprouting compared to control featured in (Fig. 2).

Hypoxic dose dependent experiments were performed on zebrafish embryos revealing early and sustained increase in development of blood vessel formation. Significant increase of blood vasculature was observed in zebrafish embryos exposed to 0.5, 1 and 2 μM $CoCl_2$ for 72 hpf compared to normoxic conditions, while embryos exposed to 3 μM or higher concentration of $CoCl_2$ exhibited necrosis or abnormal embryogenesis.

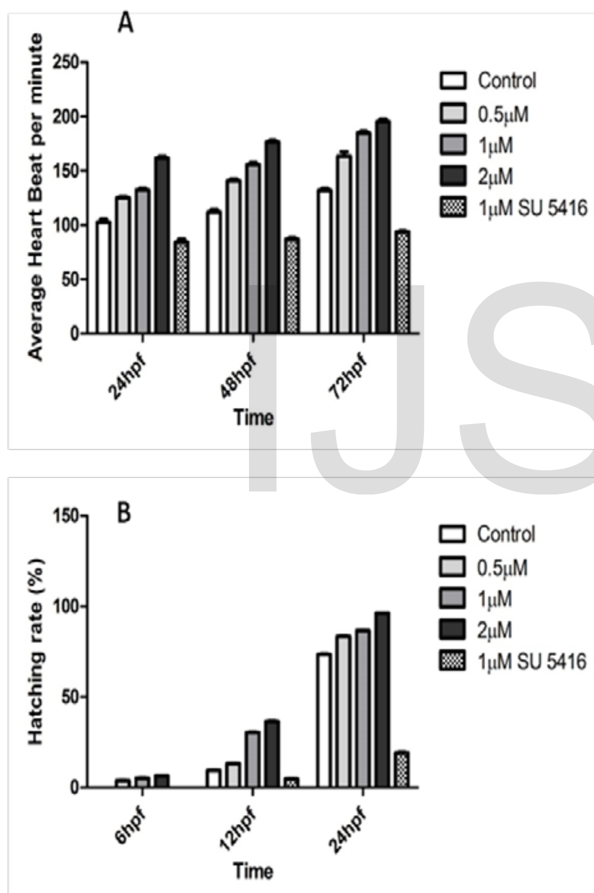

Fig. 1: A, 0.5, 1, 2 μM $CoCl_2$ exposed embryos exhibited enhanced hatching efficiency at 6, 12, 24 hpf significantly compared to control embryo. B, $CoCl_2$ treated zebrafish embryos at 24, 48 and 72 hours post-fertilization (hpf) showing increased heart beat rate. Treatment with 1 μM SU 5416 serving as positive control.

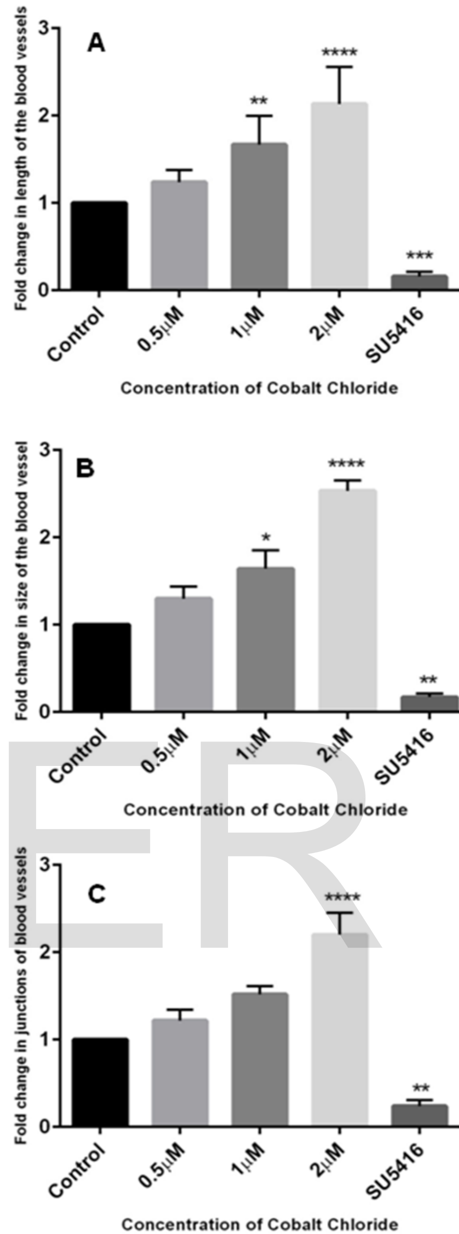

Fig.2: Fold change in $CoCl_2$ treated embryos showing significant increase in (A) length of the blood vessel, (B) size of the blood vessel, (C) blood vessel junction at various concentration of $CoCl_2$ compared to control and 1μM SU 5416, as positive inhibitor of VEGF signaling.

3.2 $CoCl_2$ induction of embryogenesis

Zebrafish embryos receiving molecular grade water (vehicle) or control caused no alteration during development (Fig.3),whereas on treating with 0.5, 1 and 2 μM of $CoCl_2$, observed a significant increase in

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length and size of the blood vessel, vasculature, vessel densities, as shown in (Fig.2), with increasing concentrations of $CoCl_2$. 1 μM of SU 5416 treated embryos shows decline in vasculature and blood vessels formation under hypoxic condition (Fig. 2).

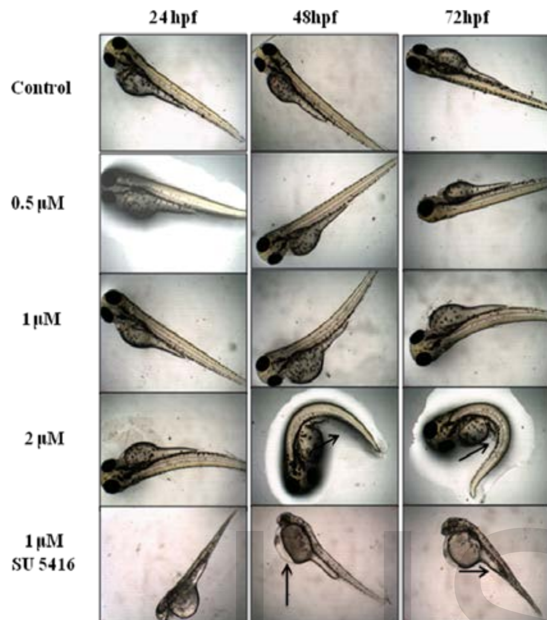

Fig. 3: Morphology of 0.5, 1, 2 μM $CoCl_2$ treated zebrafish embryos at 24, 48 and 72 hours post-fertilization (hpf). $CoCl_2$ exposed embryos exhibited curvature at 1 μM, tail bending & curvature at 2 μM compared to control embryo. Pericardial edema is observed with treatment of 1 μM SU 5416 serving as positive control.

Alkaline phosphatase staining (Fig.4) employed to highlight the presence of key architect of angiogenesis namely intersegmental vessels (ISV) in $CoCl_2$ treated embryos. Images from zebrafish embryos treated were taken on 72 hpf. $CoCl_2$-exposed zebrafish embryos were showing significant ISV formation from time of exposure than in the control zebrafish embryos developed normally (Fig. 4). The intact embryos of the $CoCl_2$-exposed zebrafish embryos displayed no signs of necrosis with doses up to 2μM, suggesting that zebrafish are less sensitive to $CoCl_2$ at lower extent compared to other hypoxic inducing compounds.

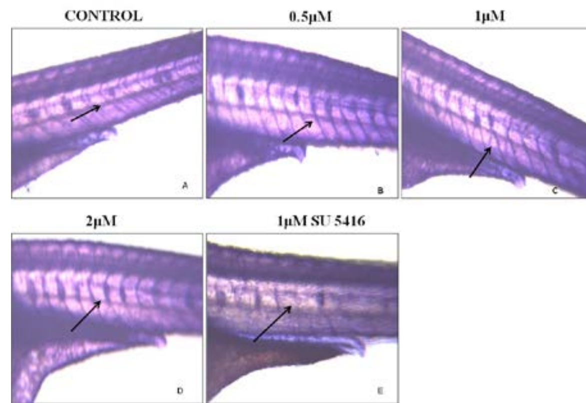

Fig. 4: APS stained zebrafish embryos at 72 hpf after $CoCl_2$ treatment. Increased ISV formation was compared with (A) control (B) 0.5 μM,(C) 1μM,(D) 2 μM (indicated by arrows).Treatment with (E) 1μM concentration of SU 5416 caused reduction of ISV formation (indicated by the arrow).SU5416 blocks key angiogenic vessel formation in zebrafish embryos.

$CoCl_2$ treated zebrafish embryos at 2 μM increased blood vessel and ISV formation compared to control after 72hpf. The same experiments were performed under 2μM $CoCl_2$ in the presence of 1 μM SU 5416 completely inhibited blood vessel (Fig. 5) and ISV development (Fig. 4). Taken as a whole, all zebrafish embryos under treatment survived healthy. Zebrafish embryos exposed to 0.5, 1 and 2 μM of $CoCl_2$ showing increase in ISV's formation with more number of blood detected by RBC staining (Fig. 5) in comparison to control developing normal vasculature whereas treatment with 1 μM SU 5416 under 2 μM $CoCl_2$ inhibited ISV and depleted RBC formation.

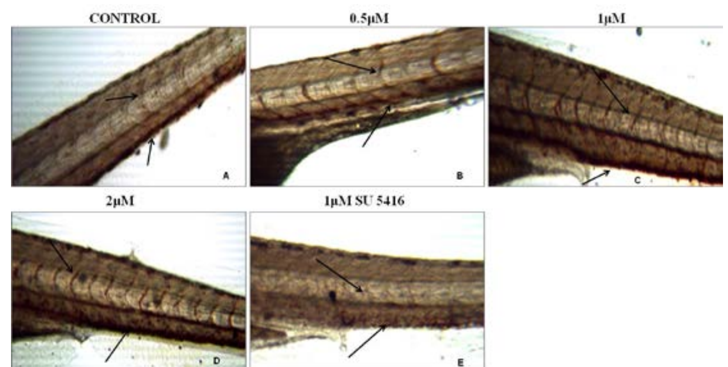

Fig.7: Angiogenic activity of $CoCl_2$ in zebrafish embryo. Lateral view of RBC stained zebrafish embryos at 72 hpf showing increased blood vessel formation with increasing concentration $CoCl_2$. (A) control, (B) 0.5µM, (C) 1 µM, (D) 2 µM. Treatment with (E) 1µM concentration of SU 5416 inhibited blood vessels (indicated by the arrow).

In treated zebrafish embryos with $CoCl_2$ at 0.5, 1µM, and 2 µM 72hpf marked significant increase in VEGF-A,VEGF R2 and HIF 1α expressions evident by qPCR and is regulated under hypoxic condition compared to control (Fig. 6). On treatment with 1 µM of SU 5416, there was considerable reduction in VEGF and VEGFR2 expression but no visible change in HIF1α expression. The morphometric analysis of the same treatment caused considerable defects in the embryo (Fig. 3) signifying the pivotal role of VEGF signalling in developmental angiogenesis.

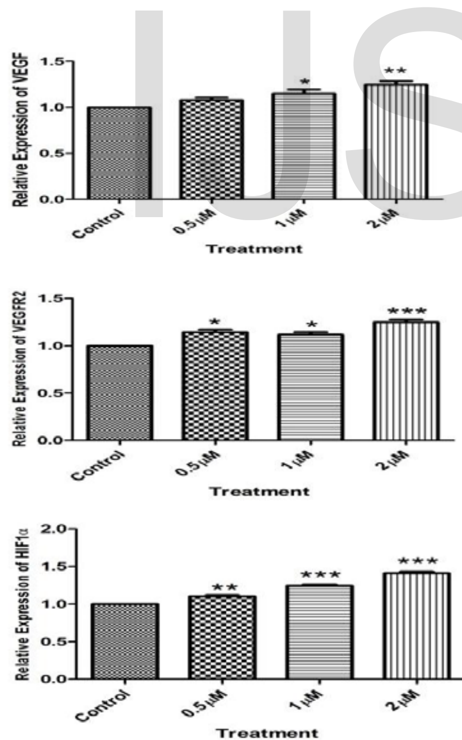

Fig. 6: Dose-response of $CoCl_2$ on the expression of VEGF mRNA in zebrafish embryos. β–actin was used as internal control. The expression levels are first normalized to β-actin and then expressed as the percentage of control. 2µM of $CoCl_2$ treated embryos up regulated VEGF-A expression when compared to that of control The data were analysed by one-way ANOVA followed by Tukey's multiple comparison tests for comparison between treatment values and control. P values < 0.05 were considered to be statistically significant. * P value <0.05, * P value <0.005, *** P value < 0.001

Western blot analysis confirmed that hypoxia-induced angiogenesis was increased under $CoCl_2$ treatment. Inhibition of the VEGF receptor or signalling by SU 5416 under hypoxic condition shown VEGF signaling is involved in hypoxia induced angiogenesis (Fig.5).

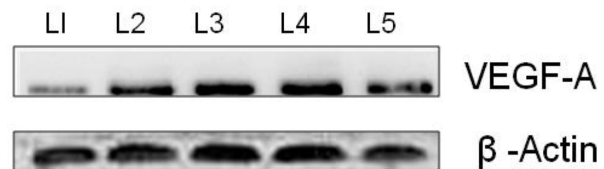

Fig. 7: Effects on VEGF Expression in Zebrafish embryos. L 1 - 1µM SU 5416, L 2 – 0.5µM, L 3 - 1µM, L 4 2µM, L 5- Control. The up-regulation of VEGF- A was confirmed by blotting analysis. The data indicated that $CoCl_2$ exerted angiogenic actions possibly via up- regulation of VEGF-A expression in Zebrafish embryos. β-actin is used as internal control.

Overall, these results suggest that $CoCl_2$ induce blood vessel in a proper fashion specifically at lower concentration to developing embryos. It also suggests that this angiogenic effect is caused by the modulation of key angiogenic players especially VEGF and VEGFR2. $CoCl_2$ is known to enhance HIF1α, as our results also suggests the same, this enhanced angiogenesis is due to the hypoxic mimentic role of $CoCl_2$ through HIF1α mediated VEGF signalling and $CoCl_2$ do modulate VEGF signalling during the process of developmental angiogenesis of embryos in zebrafish.

4. Discussion and Conclusion

Vasculogenesis and angiogenesis plays important role during development. Specific pattern of vasculature and circulatory system is necessary for survival of embryos. Zebrafish has become powerful tool to screen teratogenic effects of chemicals or drugs [12-14] .The study accelerates the potential significance of $CoCl_2$ on zebrafish embryos. Molecular mechanism underlying hypoxia mediated angiogenesis is still under cover. Angiogenesis a complex process involves vascular endothelial growth factor (VEGF), VEGF-R2 receptor mediated tyrosine kinase (RTK's) lead to ripening of endothelial cell proliferation [15-16].

Abnormal angiogenesis can lead to various disorders like tumor, macular retinopathy [17,18]. Many tumors are accelerated under hypoxic condition due to abnormal angiogenesis [19]. As $CoCl_2$ is a hypoxic mimentic agent and have shown to activate hypoxic signalling [20, 21].

Currently major gap on mechanism of $CoCl_2$ among researchers is of great concern. Current reports proposing $CoCl_2$ has bias nature, inducing and inhibiting angiogenesis. Recent data suggest the higher affinity of $CoCl_2$ towards heme group on exposure might seize ascorbate in the cells required for iron transition from $Fe3+$ to $Fe2+$ [21]. Reports on propyl hydroxyl dehydrogenase (phd's) inhibition sparked $CoCl_2$ as a hypoxic mimicking agent [22] Cobalt is proposed as inhibitor of phd to increase more number of endothelial progenitor cells, a strategy to overcome EPC transplantation [23]. Upcoming reports promotes cobalt ~0.1 μg daily is required for health as a dietary mineral [24].A reference dosage of 0.03mg cobalt per kg of body weight per day is considered as health benefactor in non cancer patients [25].It is evident from the literature that cobalt do have a role in the normal functioning of the body and the current study is of worthwhile to conduct to decipher its novel significance in context of development of embryos zebrafish is widely accepted model for developmental biology. Exposing zebrafish embryos to various metals were already screened for toxicity, in which cobalt is less specific to copper and nickel on its survival and hatching [6]. Our results suggest that $CoCl_2$ increased hatching rate, heart beat rate with enhanced blood vasculature and ISV formation in zebrafish embryos. Pericardial edema was observed in $CoCl_2$ treated embryos with higher concentration exceeding 2 μM. Blood vessels were quantified using angioquant software [8] to measure length, size and sprouting of blood vessel. Using this technique it is possible to demonstrate blood vessel formation in a right stream, revealing $CoCl_2$ can also play positive role to induce angiogenesis at low concentration, while a wrong pattern of abnormal vasculature is formed at higher concentration leading to necrosis of the cells. To further confirm hypoxic mediated VEGF expression, potent inhibitor of VEGF, SU 5416 treated under 2μM of $CoCl_2$ completely suppressed blood vessel, ISV formation indicating the importance of VEGF in angiogenesis. The data suggest the positive role of $CoCl_2$ by inducing angiogenesis under hypoxic condition. Increase in angiogenic response to hypoxia *via* HIF-1 α mechanism is still under debate. From the results obtained above, VEGF upregulation under hypoxic condition through HIF's associated signaling pathways requires more attention to study sprouting angiogenesis during wound healing, tissue repair, regeneration and development.

Thus our study demonstrates $CoCl_2$ induce angiogenesis in *"in vivo"* zebrafish model reporting increased VEGF expression at transcription and translational level with corresponding increase in HIF-1 α expression might be one of the signaling mechanisms.

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Conflict of Interest

The authors declare no conflict of interest.

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References:

[1] Chris Jopling, Guillermo Suñé, Adèle Faucherre, Carme Fabregat and Juan Carlos Izpisua Belmonte.Hypoxia Induces Myocardial Regeneration in Zebrafish. *Circulation.* 2012;126:3017-3027.

[2] Ferrara, N and Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. Nat. Med.1999; 5, 1359 – 1364.

[3] Carmeliet P. Mechanisms of angiogenesis. Nat. Med., 2000; 6: 389-395.

[4] Peterson RT, Link BA, Dowling JE and Schreiber SL .Small molecule development screens reveal the logic and times of vertebrate development, Proc.Natl Acad Sci USA,2000;97(24): 12965-9.

[5] TinChung Leung, Hui Chen, Anna M. Stauffer, Kathryn E. Giger, Soniya Sinha, Eric J. Horstick, Jasper E. Humbert, Carl A. Hansen, and Janet D. Robishaw .Zebrafish G protein γ is required for VEGF signaling during angiogenesis. Blood.2006;108(1);160-6.

[6] Dave G and Xiu RQ. Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. Arch Environ Contam Toxicol.1991;21(1):126-34.

[7] Tonang dwi Ardyanto, Mitsuhiko Osaki, Naruo Tokuyasu,Yumi Nagahama and Hisao Ito. $CoCl_2$-induced HIF-1· expression correlates with proliferation and apoptosis in MKN-1 cells: A possible role for the PI3K/Akt pathway. International Journal of Oncology 2006; 29: 549-555.

[8] Antti Niemisto, Valarie Dunmire, Olli Yli-Harja, Wei Zhang and Illya Shmulevich. Robust quantification of in vitro angiogenesis through image analysis. IEEE Transactions in Medical Imaging, 2005; 24(4): 549-553.

[9] Lowry LH, Rosenbrough JN, Farr AL and Randall RJ. Protein measurement with the Folin Phenol Reagent. J Biol Chem. 1951; 193(1): 265-75.

[10] Iuchi I and Yamamoto M. Erythropoiesis in the developing rainbow trout, Salmo gairdneri deus: histochemical and immunological detection of erythropoietic organs. J. Exp. Zool 1983: 226, 409-417.

[11] Nicoli S and Presta M. The zebrafish/tumor xenograft angiogenesis assay. Nat. Protoc. 2007, *11*, 2918–2923.

[12] Fraysse B, Mons R and Garric J. Development of a zebrafish 4-day embryolarval bioassay to assess toxicity of chemicals. Ecotoxicol Environ Saf, 2006;63:253–67.

[13] Selderslaghs IW, Van Rompay AR and De Coen W. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod Toxicol 2009; 28:308–20.

[14] Yang LX, Yang L and Ho NY. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod Toxicol 2009; 28:245–53.

[15] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr. Rev. 2004; 25: 581–611.

[16] Ferrara N. Timeline: VEGF and the quest for tumour angiogenesis factors. Nat. Rev. Cancer 2002; 2:795–803.

[17] Semenza GL, Roth PH, Fang HM, and Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem. 1994; 269, 23757–23763.

[18] Gasparini G and Harris AL. Clinical implications of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool, Journal of clinical oncology, 1995; 13:765-82.

[19] Salnikow K, Davidson T, Zhang Q, Chen LC, Su W, and Costa M. The involvement of hypoxia-

inducible transcription factor-1-dependent pathway in nickel carcinogenesis. Cancer Res 2003; 63, 3524–3530.

[20] Mace KA, Yu DH, Paydar KZ, Boudreau N and Young DM. Sustained expression of Hif-1alpha in the diabetic environment promotes angiogenesis and cutaneous wound repair. Wound Repair Regen 2007; 15:636-45.

[21] Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, and Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress.J. Biol. Chem.2004; 279(39); 40337-44.

[22] Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, and Ratcliffe PJ. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001; 107, 43–54.

[23] Knowles HJ, Tian YM, Mole DR and Harris AL. Novel mechanism of action for hydralazine: induction of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and angiogenesis by inhibition of prolyl hydroxylases. Circ Res 2004; 95:162-9.

[24] Finley BL, Monnot AD, Paustenbach DJ and Gaffney SH. Derivation of a chronic oral reference dose for cobalt. Regul Toxicol Pharmacol. 2012; 64: 491–503.

[25] Bradberry SM, Sabatta P and Vale JA, [Monograph] 1989. [http://www.inchem.org/documents/ukpids/ukpids/ukpid50.htm]

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