ORIGINAL ARTICLE

Eur. J. Anat. 24 (3): 211-220(2020)

Late administration of caffeine affects cardiac maturation in chick embryos: a combined two and three dimensional morphogenetic and gene analyses

Nichapha Chandee[1,2], Nutmethee Kruepunga[1], Thanyarat Leckchaoum[1], Pakpoom Thintharua[1], Wattana Weerachatyanukul[1], Somluk Asuvapongpatana[1]

[1] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, Thailand
[2] School of Allied Health Science, Walailak University, Nakhon Si Thammarat, Thailand

SUMMARY

Cardiac malformations are very prevalent and can be caused both by defective genes and environmental teratogens. Among the latter, caffeine causes malformations when exposed during early cardiac development, whereas its later effects are still unclear. We exposed three-day incubated (D3) chick embryos to 2 mg caffeine and analyzed them at D5, D7 and D9. The embryos were serially sectioned and analyzed two-dimensionally.

Alternatively, the sections of D9 embryos were reconstructed three-dimensionally using Amira® software and analyzed volumetrically. The expression of genes involved in endothelial-mesenchymal transformation (EMT) was studied by real-time PCR. Interestingly, caffeine treatment at D3 embryos did not induce cardiac malformations, but did delay growth, in particular that of the ventricles and ventricular trabeculae. Furthermore, it affected EMT in the endocardial cushion and atrioventricular valves. Gene-expression analysis revealed that caffeine had a progressively deleterious effect on the expressions of GATA4, MMP2, SNAIL1, TWIST1, and VIMENTIN. The effect of late caffeine administration on the chicken embryos would provide suggestive evident towards a possible heart developmental defect in humans, particularly heavy caffeine consumers during pregnancy.

Key words: Chick embryo – Cardiac development – Cardiac maturation – Ventricular defect – Caffeine – 3D analysis

INTRODUCTION

Recent advances in methodologies to unravel successive events in organ development often rely on 3D image reconstruction. This approach offers a clearer image of the spatial and temporal changes in the development of organs (Carlson, 1981; Hopwood, 2002). Together with gene expression analysis, they provide powerful tools to gain insight into critical developmental events. From these approaches, several genes and their translational proteins have emerged to play important roles in regulating successive steps of cardiac development. Two categories of genes are often distinguished: 1) those that govern cardiac patterning, and 2) those that reflect cardiac maturation (Fuster and Walsh, 2011). Genes that regulate cardiac morphogenesis belong to the first category (Yelon, 2001), whereas genes that are involved in the es-

Corresponding author: Assoc. Prof. Somluk Asuvapongpatana. Department of Anatomy, Faculty of Science, Rama 6 Rd., Ratchathewi, Bangkok 10400 Thailand .
E-mail: somluk.asu@mahidol.ac.th

Submitted: 28 January, 2020. *Accepted:* 20 March, 2020.

tablishment of functional components of the heart belong to the second category (Kolanowski et al., 2017). Examples are the well-known cardiac specification factor TGF-β that is synthesized and secreted by hypoblastic cells to induce the formation of cardiogenic mesoderm, which then extends towards the lateral plate under the influence of BMP2 and FGF8 (secreted by the underlying endoderm) to become the cells of the first heart field (Brand, 2003; Evans et al., 2010; Galdos et al., 2017). These committed cardiogenic cells express NKX2.5, GATA4 and TBX5 (Ban et al., 2013; Bruneau, 2013; Zaffran and Frasch, 2002). The more caudal part of the cardiogenic plate forms the second heart field, with TBX18 and ISL1 as patterning genes (Cai et al., 2003; Hoogaars et al., 2007) and FGFs as mediators of maturation (Kelly et al., 2001). HNK1 is a factor that regulates neural crest cells, yet another source of cardiac cells (Creazzo et al., 1998) to form cardiac outflow tract (OFT) (Luider et al., 1993).

Cardiac maturation factors play a role in several steps during late cardiac development, including chamber maturation and valve formation (Dunn and Palecek, 2018; Samsa et al., 2013). Chamber maturation, both atria and ventricles, require the coordinate expression of many genes, including GATA4, TBX5, NKX2.5, IRX4, MEF2C, HAND1, and HAND2 (Brand, 2003; Bruneau, 2002; Kelly, 2012; Paige et al., 2015). Cardiac valve formation is associated with epithelial to mesenchymal transition (EMT) (Runyan et al., 2013), starting with reorganization of endocardial cushion by remodeling enzymes, of which MMPs 1, 2 and 13 promote cell migration, while increased cellularity and collagens provide rigidity to the developing valves (Combs and Yutzey, 2009). Other important factors that are associated with EMT during valve development include SNAIL and TWIST1, which are the zinc-finger E-box binding (ZEB) transcription factors. These factors are activated at the beginning of EMT upon accumulation of vimentin (Liu et al., 2015).

Recently, several studies have shown that caffeine over-consumption in pregnant women can interrupt embryonic or fetal heart development (Chen et al., 2014). In mice, early caffeine exposure can lead to a decreased thickness of the ventricular wall, OFT defect, and defective atrioventricular (AV) valve formation (Wendler et al., 2009). While early exposure to caffeine (Day 1 embryo) affects many structures in the developing heart, the effects of a late exposure of caffeine on cardiac development remain to be demonstrated. We experimentally mimicked the situation by exposing Day 3 chick embryos (equivalent to Carnegie stage (CS) 14 of human embryos (about 34 day of development) (Butler and Juurlink, 1987; O'Rahilly and Muller, 2010) to a single dose of caffeine in this study. Our combined 2D and 3D image analyses clearly showed that this exposure did not induce any morphological malformation in the developing heart, but that the functional maturation of ventricles was delayed. This finding thus raises concern about adverse effects of caffeine consumption during pregnancy.

MATERIALS AND METHODS

Animals and caffeine treatment

Day-3 Leghorn chicken embryos were obtained from the Suwan Vajokkasikit farm at Kasetsart University. Animal handling was approved by the Animal Care and Use Committee, Faculty of Science, Mahidol University (SCMU-ACUC, protocol no. MUSC61-029-431). Chicken embryos were either injected with a single dose of 2 mg caffeine/egg (a LD50 dose, N=80, only survival embryos), or with solvent (0.9% NaCl, N=20). Before caffeine injection, the pre-incubated eggs were disinfected with 70% ethanol and a hole was drilled in the egg shell without damaging the embryonic disc. Then, 200 µl of ovalbumin was removed before injection of 100 µl 0.9% NaCl or caffeine solution. The hole in the egg shell was sealed with paraffin film and eggs were further incubated at 37°C and 80% humidity for 2, 4 and 6 days to allow analysis of the embryos at D5, D7, and D9, respectively. All of the embryos in caffeine-treated and control groups were subjected to either RNA extraction for real-time PCR (N= 5) or histological processing for 2D- and 3D-image analysis (N=5 each).

Tissue processing

The incubated eggs were cracked and transferred to avian Ringer's solution. The embryonic discs were dissected, cleaned with PBS and fixed with Bouin's solution for 24 hr. The fixed tissues were then dehydrated in serially graded ethanol, cleared in xylene, and embedded in paraffin at 60° C under vacuum in the Leica TP1020 Semi-enclose Benchtop tissue processor. The tissue blocks were serially sectioned at 8-10 µm thick and stained with hematoxylin and eosin. The sections were digitized with an Olympus VS120 virtual slide scanning system attached to an Olympus BX53 microscope.

Two-dimensional image analysis, 3D reconstruction and volumetric analysis

We initially analyzed 2D-morphometric parameters in coronal sections of caffeine-treated and non-treated D9 embryos. Sections selected for 2D morphological analysis had to meet three structural criteria: (i) presence of four heart chambers; (ii) presence of at least two leaflets of both left and right atrioventricular valves; (iii) presence of at least four large arteries, including aorta, subclavian artery, common carotid artery and brachiocephalic trunk (see Fig. 1). The following morphometric parameters were measured: thickness of ventricular myocardium, length of valve leaflets, and valvular

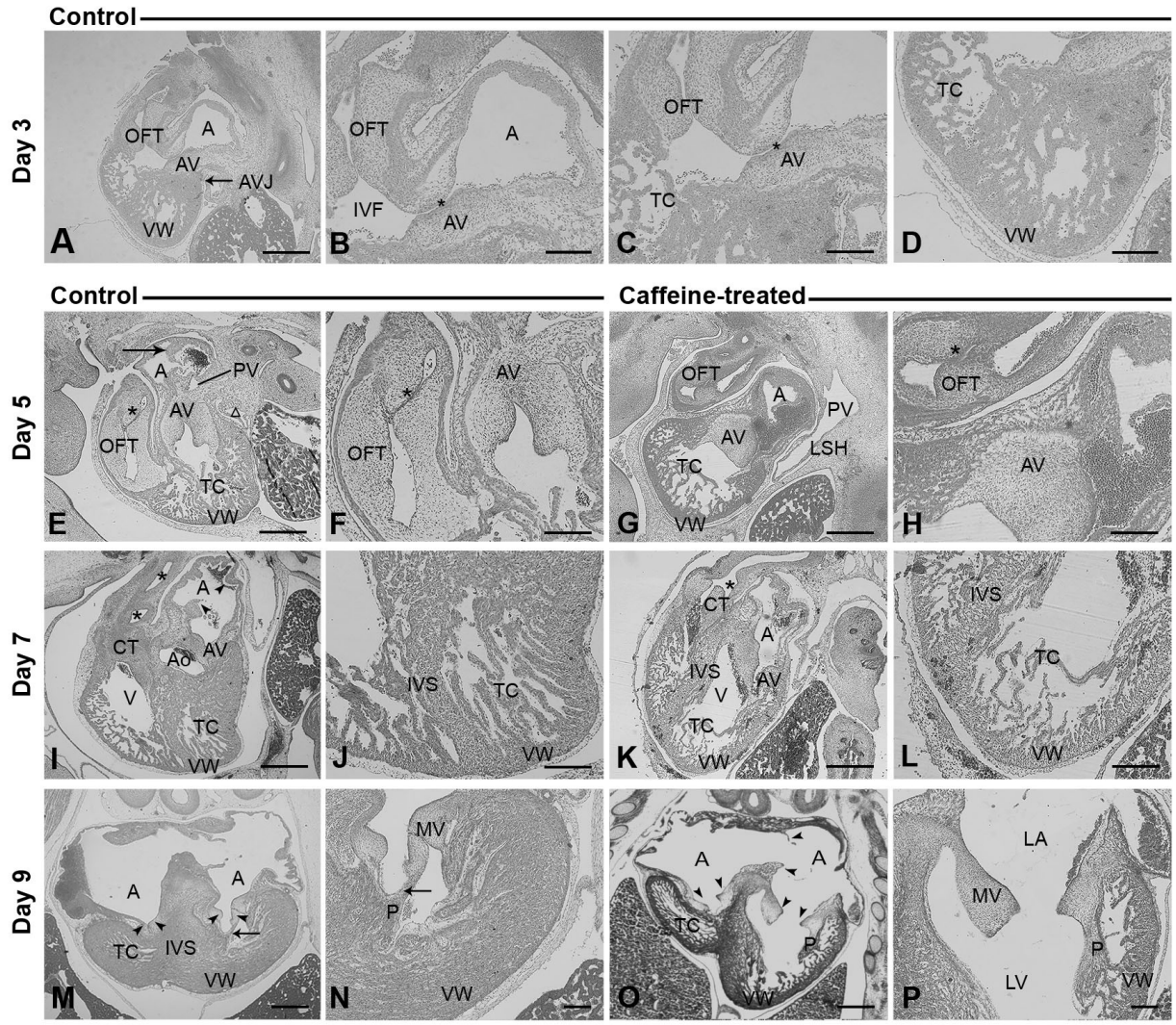

Fig. 1.- Cardiac phenotype in embryos treated with caffeine on D3. Non-treated embryos were studied at day 3 (**A-D**) day 5 (**E-F**), day 7 (**I-J**) and day 9 (**M-N**) and caffeine-treated animals at D5 (**G-H**), D7 (**K-L**), and D9 (**O-P**). Three features were prominent in caffeine-treated hearts: thinner ventricular wall (VW), lower cell density in endocardial cushions, and retarded valve development with absent papillary muscles at D9 (arrowheads). A: atrium; Ao: aorta; arrow: atrioventricular junction (A), chordae tendineae (N); AV: atrioventricular cushion; CT: conotrancal; IVF: interventricular foramen; LA: left atrium; LSH: left sinus horn; LV: left ventricle; MV: Mitral valve; OFT: outflow tract cushion; P: papillary muscle; PV: Pulmonary vein; TC: trabeculae carneae; VW: ventricular wall; V: ventricle. Scale bars in F, H, J, L, N, P = 200 µm, A-E, G, I, K = 500 µm, M and O = 1000 µm.

cell density. The thickness of the compact ventricular myocardium was measured at 5 randomly selected areas per embryo in 5 different embryos (25 areas in total). The length of valve leaflets both left and right valves was measured proximally from the boundary line of mesenchymal condensation towards distal tip of valve leaflets. The valvular (endocardial-derived) cell density was estimated with Olympus CellSens software after staining of cell nuclei and reflected number of cells per μm^2 in the randomly chosen endocardial mesenchymal areas. The ventricular compact wall and lumen were manually segmented and calculated with the Material Statistics module in Amira 3D. All statistical data were analyzed by SPSS17 (IBM corporation, Armonk, NY).

RNA isolation and RT-PCR

Gene expression levels were analyzed by a real-time PCR, which followed the conditions described by Roche experimental protocol. RNA was extracted by homogenizing in Trizol and ethanol precipitation immediately after sample collection. Total RNA concentration was measured at 260/280 nm, using a NanodropTM 2000/c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and stored at -80°C. The RNA was converted into cDNA using a Promega cDNA reverse transcription kit (Promega, Madison, WI). The primer pairs for several markers of cardiac maturation (GATA4, SNAIL1, TWIST1, MMP2, VIMENTIN) and β-actin (internal control) were designed from the Gallus gallus gene sequences in the NCBI database (Table 1). The qPCR was performed in the iCycler

Table 1. Primers used for cardiac maturation markers

Primers	Forward sequence 5' 3'	Reverse sequence 5' 3'
GATA4	TCAACCGGCCCCTCATTAAG	GTGGTGGTAGTCTGGCAGT
SNAIL1	CACACGCTGCCTTGTGTCT	GGTCAGCAAAAGCACGGTT
TWIST1	TTCCGAATTTGCCTGTTTTT	GTTGGGTGCTTTGCTTTCAT
MMP2	GATGTCGCCCCTAAAACAGAC	CAGCCATAGAAAGTGTTCAGGT
VIMENTIN	AGGAAATGGCTCGTCACCTTCGTGAA-TA	GGAGTGTCGGTTGTTAAGAACTAGAGCT
β-actin	AAACTGGAACGGTGAAGGTG	AGAGAAGTGGGGTGGCTTTT

iQ real-time PCR detection system (Bio-Rad, København, Denmark) using SYBR green dye. PCR mixture included 10 µL of Luna Universal qPCR Master Mix, 0.5 µL forward and reverse primer, 0.5 µL cDNA templates, 20 µL nuclease-free water. The thermal cycling conditions included an initial denaturation at 95°C for 1 min, 45 cycles of denaturation at 95°C for 15s, annealing with gradient temperature optimization at 48.7-57.7°C for 1 min, extension at 60°C for 30s and melt curve with 60° C for 1 cycle were carried out. Real-time PCR reactions were carried out in triplicate for each sample and the data were expressed as mean±S.E.

Data Analysis

Data of all parameters collected from 2D- and 3D-images analysis (N=25 from 5 embryos) were expressed as means ± S.E. and further analyzed using a Student paired t-test between the control and 2 mg caffeine/egg samples. The results of real-time PCR (N=3) for all markers of maturation were expressed as means ± S.E., and the graphs were plotted by a GraphPad Prism 5.0 software package (GraphPad Software, San Diego, CA), and further analyzed using a Paired t-tests. The relative change of gene expression was calculated by comparing individual gene expression value in the caffeine-treated group to the referencing value of control group at the given incubation days. In all statistical comparisons, P-value of <0.05 indicated a significant difference.

RESULTS

Caffeine-induced morphological changes in the developing chick heart

We studied sections of chicken embryos at different time points after the exposure to 2 mg caffeine. At day 3 of incubation (D3), heart looping is nearly complete, while the atrial and ventricular chambers have become well recognizable (Fig. 1A, B) and separated by an atrioventricular (AV) canal of considerable length (*). The atrial chamber (A) was a common chamber still without the separating primary atrial septum. Thickening of AV cushions along the AV canal was clearly noted above the inlet of ventricle (Fig. 1C), whereas the right ventricle formed the ventricular outlet that was connected with the pharyngeal arch arteries via the outflow tract (OFT). The OFT was a single tube on the right hand side of the atrium that was lined with thick mesenchymal tissue (endocardial cushions). A ventricular chamber was still a single chamber in which its wall (VW) was lined by primary myocardium at this stage (Fig. 1D). The trabeculae (TC), which project from the myocardial wall, gave the ventricles a distinct morphology.

At D5 of normal development, the cavity of primary atrium increased in size and contained the venous valves (Fig. 1E, arrow), while the pulmonary vein (PV) approached the future left atrium. The mesenchymal tissue of AV canal was pronounced and fused (Fig. 1F). Definitive septation to form aorticopulmonary septum occurred from downstream to upstream in the OFT (Fig 1F, *). The ventricular walls were lined extensively with trabeculae carneae (TC). Two days post-injected D3 embryos with caffeine (D5), heart development was somewhat delayed with similarities to the D3 atrium (Fig. 1G). Although the AV cushions had fused, they lacked the downward mesenchymal extensions (compared Fig. 1H and 1F, AV). The thickness of primary myocardium was similar to that in the control, but the number of trabecular projections was markedly less (Fig. 1G). The OFT morphology was similar to that of the control (Fig. 1H, *).

On the 7th day of normal development, the atrial cavity had expanded and the primary interatrial septum had extended from the roof of the atrium (Fig. 1I, arrow heads). The AV cushions had fused, forming the separated left and right atrioventricular channels. The left and right ventricles were clearly separated by an interventricular septum. Trabeculae carneae (TC) filled a large part of the lumen of the ventricles (Fig. 1J). Upon treatment with caffeine for 4 days (D7), the atrial chamber appeared less expanded and the interatrial septum appeared shorter (Fig. 1K). The AV cushions were fused, but their cell density was less. The interventricular septum (IVS) had formed as in the controls (Fig. 1K), but the trabeculae carneae were shorter and thinner than those in the controls (Fig. 1L). The development of the OFT appeared normal, namely, there was a presence of aorticopulmonary septum in developing OFT.

In the control D9 heart, the atria were separated

Table 2. Morphogenetic analysis of the 2D and 3D parameters of the control and caffeine-treated embryos

2D-Parameters		
Parameters	**Untreated Embryos**	**Treated Embryos**
Atrial thickness (Rt) (μm)	25.91±1.45	23.74±2.79
Atrial thickness (Lt) (μm)	34.99±2.70	22.86±2.47
Ventricular thickness (Rt) (μm)	260.18±10.80	78.40±5.43*
Ventricular thickness (Lt) (μm)	352.25±16.97	124.77±10.20*
Length of valve leaflets (Rt) (μm)	280.62±15.07	502.89±33.93*
Length of valve leaflets (Lt) (μm)	395.75±15.47	542.88±31.05*
Endocardial cell density (Rt) (cells/μm^2)	1836.2±247.36	1543.3±304.8
Endocardial cell density (Lt) (cells/μm^2)	1700.5±304.68	1602.6±300.56
3D-Parameters		
Parameters	**Untreated Embryos**	**Treated Embryos**
Atrial wall (μm^3)	19.7×10^9	7.9×10^9
Ventricular wall (μm^3)	45.4×10^9	11.5×10^9
Ventricular lumen (μm^3)	10.7×10^9	8.3×10^9

* P-value ≤ 0.05 are considered statistically significant (paired t-test)

by the primary interatrial septum that attached to the endocardial cushions (Fig. 1M, arrow heads) and had formed a secondary foramen. The AV canal was separated by fused AV cushions forming right and left atrioventricular valves (Fig. 1M). Papillary muscles were also recognizable (Fig. 1N, P). Both ventricles were well separated by a prominent interventricular septum and tightly packed TC on the thick ventricular wall (Fig. 1N). Upon treating with caffeine for 6 days (D9), the features of the atria were rather similar to those of control embryos (Fig. 1O). Both AV valves connecting atria to ventricles could be clearly defined. The underdeveloped features were more pronounced in the ventricles, including less-dense mesenchyme in the AV cushions and valve leaflets (appeared plumper than control). Furthermore, the myocardium of the ventricular wall had fewer trabeculae carneae (Fig. 1P).

Two-dimensional morphogenetic analysis

We compared the morphology of developing hearts upon treating D3 embryos with 2 mg caffeine. Comparatively, the size of hearts of D5, D7 and D9 embryos was not significantly altered between treated and control groups: 1.15 × 1.59 mm at D3, 1.11 × 1.74 mm at D5, 1.6 × 2.15 mm at D7, and 2.83 × 2.21 mm at D9. Since D9 embryos showed the most pronounced effect of caffeine on cardiac development, we carefully analyzed many areas of developing heart, including chamber formation and thickness, OFT orientation, compaction of the endocardial cushions and valvulogenesis. The analysis of the atrial wall thickness showed that wall thickness was not different, but the volume was >2-fold decreased in caffeine-treated embryos (Table 2). Furthermore, both valve leaflets on the left and right ventricles were longer, while their cell density was less in the caffeine-treated hearts. These differences reached significance at the right side only. Ventricular wall thickness was more profoundly affected: the wall thickness in the left ventricle was reduced to ~35% of control, whereas that in the right ventricle ~30%, both differences being statistically significant. The data of this morphologic analysis thus supported the histological evidence shown in Fig. 1.

Three-dimensional morphogenetic analyses

We constructed three dimensional models of D9 hearts from both groups and compared their quantitative volumetric analysis. Three dimensional models of the hearts were created from 120 coronal sections and used as representatives for volumetric analysis of the whole or selected heart areas. Since ventricular compartment was most drastically affected in the late caffeine treatment, we monitored more detailed parameters in the ventricular region and analyzed volumetric data of ventricular wall, lumen as well as endocardial cushion.

Three-dimensional volumetric analysis of the ventricle indicated that the total volume of ventricular wall was much smaller in the treated animal (11.5×109 mm^3 versus 45.4×109 mm^3, which accounted for one-fourth difference) while the volume of the lumen was only slightly different (Fig. 2C, F and Table 2). The most striking difference was a diffused architecture of the loose mesenchymal cells within an endocardial cushion in embryos exposing to caffeine while the control heart showed a clear segmentation of the mesenchymal cells into two separated clusters, presumably the future mitral and tricuspids valves (Fig. 2B, E). This still-fusion of endocardial mesenchyme could not be easily notable in the 2D coronal sections

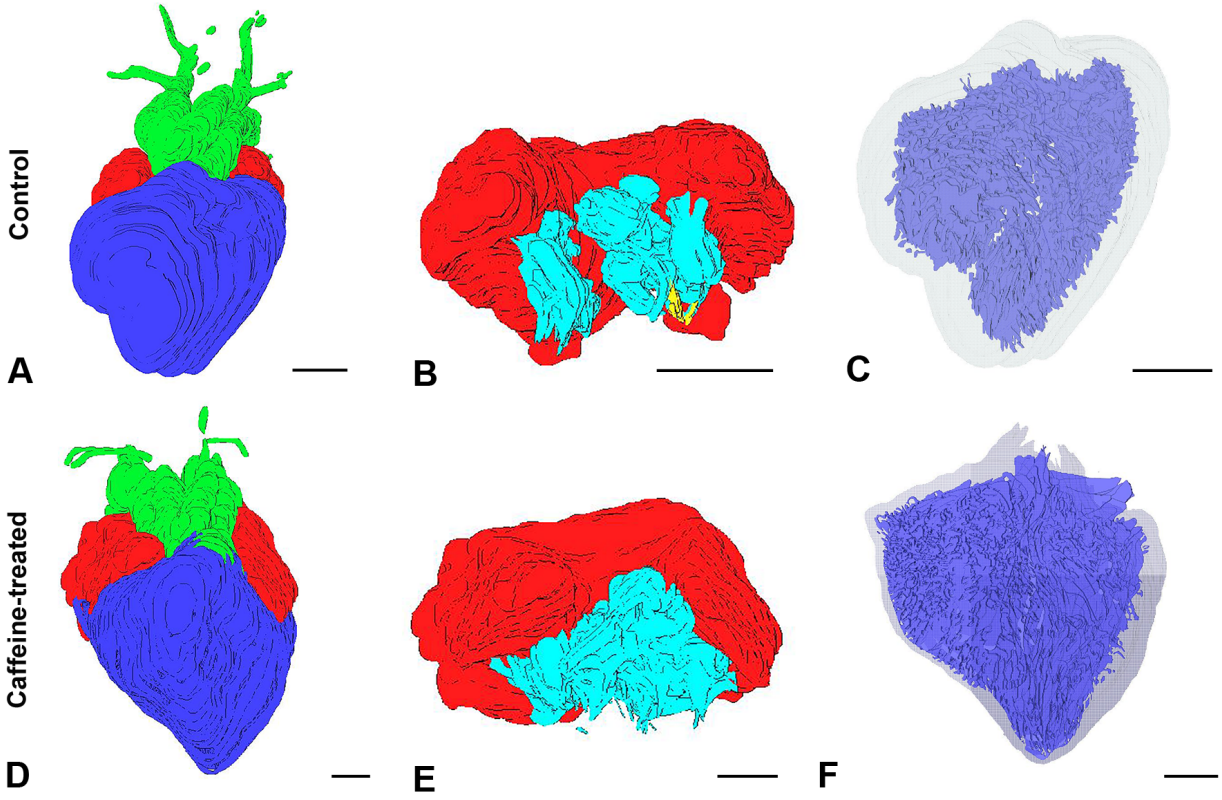

Fig 2. Three-dimensional reconstructions of the heart of a control and a 2-mg caffeine-treated embryo. The 3D volumes were generated from 120 coronal sections and the edges of each structure in the developing heart were segmented manually with Amira software. The exterior surfaces of D9 control embryo (A) and caffeine-treated embryo (D) are compared while their interior surfaces at endocardial mesenchyme and AV valves of both models are shown in panels B and E. The interior and ventricular wall thickness are represented in panels C and F. Scale bars = 1000 µm. Red: atrium; Navy blue: ventricle; Cyan: endocardial cushion; Yellow: papillary muscle; Green: cardiac outflow tract; Blueish grey: ventricular lumen.

analyzed above. We thus strongly believe that 3D image reconstruction and its volumetric analysis would give a holistic ventricular analysis and provided a good evidence of caffeine effect on ventricular defect.

Effect of caffeine on gene markers of cardiac maturation

Our morphological analysis showed that caffeine administration in late cardiac development exerted an apparent effect on cardiac development, particularly the ventricle. The developing valves stood out, because they appeared to suffer from a qualitative effect in being less populated by endocardial cells. We, therefore, analyzed a set of genes that play a key role in EMT, including GATA4, MMP2, VIMENTIN, SNAIL1 and TWIST1 (Combs and Yutzey, 2009; Liu et al., 2015). Overall, the 2 mg dose of caffeine induced down-regulation of the expression of all these genes except MMP2 and SNAIL1of D5 embryos. The most pronounced effect of caffeine treatment was observed for GATA4 and VIMENTIN, of which the expression at D7 and D9 in the caffeine-treated animals was ~20% less than that observed in the control animals ($P < 0.05$; Fig. 3A and B). The differences in expression of MMP2 and TWIST1 were less pronounced, but significant. Interesting, the decrease in expression became more pronounced with age, indicating that the pejorative effect of caffeine on heart morphology and function was progressive.

DISCUSSION

In early embryological studies, detailed analyses and explanations of developmental processes and several congenital disorders have relied mainly on either histological section (in case of animal models) or plain image sections on CT scan (in case of clinical diagnosis). One of the major problems that occur during 2D image analysis is the selection of the proper planes of the sections to be analyzed and compared between either the parallel treatment groups or control. In order to fix the plane for 2D analysis, several focal markers in the sections have to be indicated (as also performed in this study in Fig.1 and Table 1) so that the closely aligned/related sections are convincingly compared. Nonetheless, some un-intentional bias or errors do exist, particularly, in the context of plane analysis, owing to the difficulty in the choice of focal markers in the sections. Recently, 3D recons-

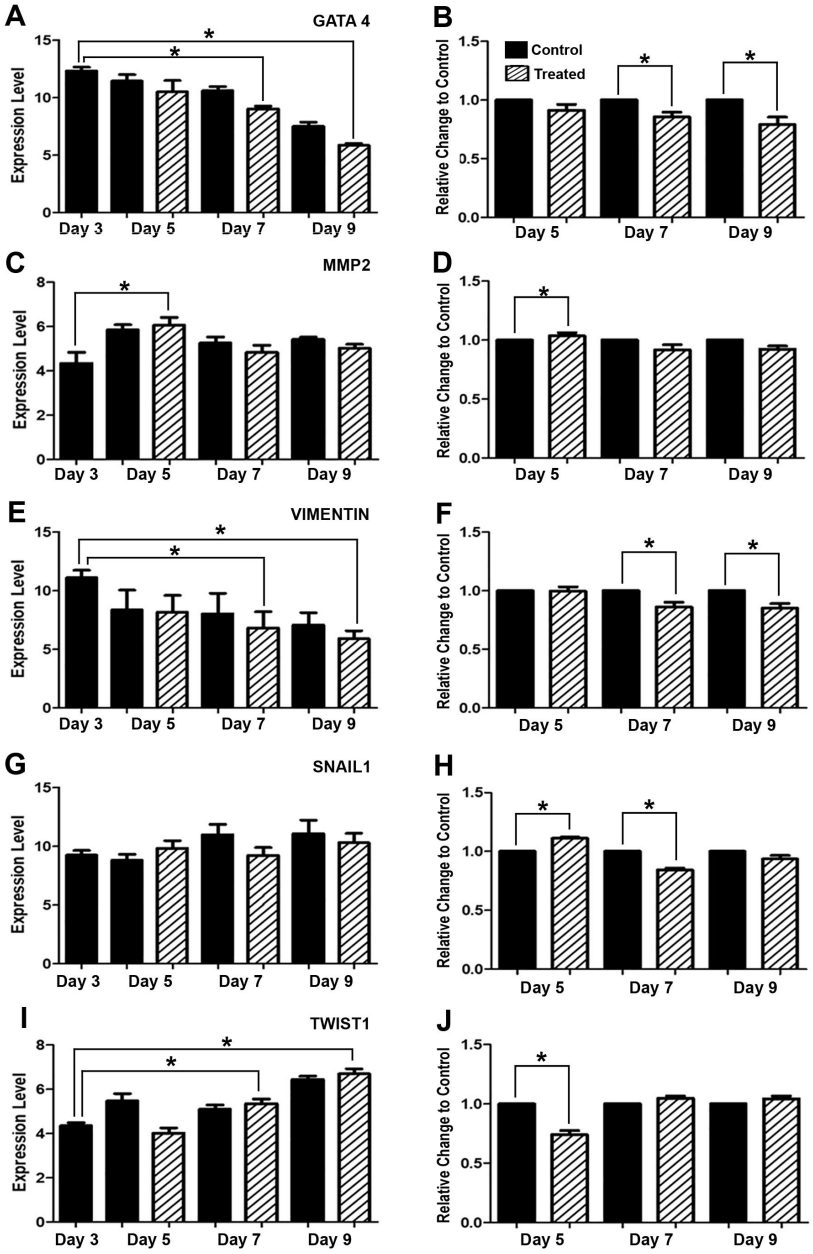

Fig 3. Quantitative measurements of gene expression in control and caffeine-treated embryos using RT-PCR analysis. The genes studied were *GATA4, MMP2, VIMENTIN, SNAIL1,* and *TWIST1*. The data were expressed as mean ± S.E. from triplicate experiments (left panels) while the relative change of the expression levels (right panels) were computed by comparing the values of gene expressions in the caffeine-treated embryos to that of the referencing values of the control embryos of the same given day. Late caffeine administration at D3 embryos had minimal effect on cardiac structural patterning, but apparently affected final structure maturation, particularly those in the ventricles including less cellular density of endocardial cushion and the associated atrioventricular valve formation as well as ventricular myocardial thickness. Caffeine also had a progressively deleterious effect on the EMT gene-expression patterns that are related to cardiac maturation including GATA4, SNAIL1, TWIST1, MMP2 and VIMENTIN.

truction plays an important role to improve the visualization of developing structures and demonstrate the realistic relationship of the entire tissue structures without any bias in plane dis-alignment. Many parameters of morphometric analyses can also be quantified to precisely describe the change in the developmental processes (Aanhaanen et al., 2010; Cavalcanti and Duarte, 2008; de Bakker et al., 2016; Engelmann et al., 1987; Kruepunga et al., 2018; Li et al., 1994). In our case, we realize that development of heart is one of the most complicated processes among many organs. Combination of both 2D and 3D analyses to monitor the changes in the late developing heart after caffeine treatment appear to be essential for data interpretation. The clear example of how 3D analysis improved the information of 2D analysis was in the part of delayed valvular development in caffeine treated animals. The results in 2D analysis demonstrated less mesenchyme density within AV

cushion and “plumper” shape of valve leaflet in the treated animals while 3D analysis could further demonstrate a still-fusing valvular complex in the treated animals while such complex was well-segmented in the control animals (Fig. 2) which otherwise could not be seen in 2D illustration. Therefore, using both 2D and 3D morphometric analyses would provide sufficient quantitative information to verify the effect of caffeine in developing heart as showed herein (Table 2) and other studies (Kruepunga et al., 2018). Our results in this study pointed out the fact that late developing chick embryos (day 3) exposed to caffeine at its LC50 dose caused an apparent delay of cardiac development, most noticeable at the ventricular wall, AV cushion and valve morphology.

Exposure of D1 chick embryos about 3 mg per egg of caffeine results in many severe cardiac malformations, and inhibit morphogenesis of organ primordia (Kobayashi et al., 1995; Lee et al., 1982). Based on our findings, we posited that the effect of late caffeine exposure favored developmental delay rather than “structural malformation” (or cardiac patterning). This claim was based on the observations of the “still-appearing” developmental structures in a delayed fashion rather than normal development. For instance, the presence of interatrial septum at D5 of normal development rather than in D7 of late caffeine-treated heart, or the complete extension of valve leaflets that were found in D9 rather than in D7 (Fig. 1). While the mechanism of delayed cardiac development caused by caffeine has not yet been established and subjected to be discussed below, the severe effect of caffeine in the model exposed earlier (D1) has been suggested to depend on two mechanisms of action during development. Caffeine is known as a phosphodiesterase inhibitor, which causes an increase in intracellular cAMP concentration (Echeverri et al., 2010), which in turn, controls genes involved in cardiac development such as GATA4, NKX2.5, and HNK-1 (Hutson and Kirby, 2007; Kim and Kass, 2017). Caffeine also acts as adenosine receptor (AR) antagonist. In the heart, the isoform of the AR is A1AR, which protects developing embryos against many harsh conditions (Buscariollo et al., 2011). Therefore, binding of caffeine to A1AR in competition with adenosine would thus directly perturb embryonic protection mechanisms, leading to many severe congenital malformations. Selectivity of caffeine towards either mode of action, which may provide a better explanation in development perturbance, has not been clearly established. Nevertheless, the two modes of caffeine action would likely share a common interfering downstream signaling, in which the levels of cardiac patterning genes such as GATA4, NKX2.5, HNK-1 are highly affected as already mentioned above for intra cAMP concentration. Similarly, perturbance of cardiac A1AR receptor by caffeine administration has also led to down regulation of cardiac patterning gene levels in conjunction with the decreased receptor level (Rivkees and Wendler, 2012, 2017).

In the case of late caffeine exposure, we observed that the genes that were affected included VIMENTIN, SNAIL1 and TWIST1 (Fig. 3) which are known to be involved in cardiac endothelial to mesenchyme transition (EMT) (Liu et al., 2015; von Gise and Pu, 2012). Since completion of the valvular complex and myocardial thickening and branching (to form trabeculae carneae) are both part of cardiac structural maturation (Combs and Yutzey, 2009; Samsa et al., 2013), we had hypothesized that late caffeine administration targets the EMT signaling cascade. Direct or indirect of caffeine inhibition to EMT process and genes has been reported, most of which are related to migration of cancer cells (Chen et al., 2014) or down regulation of EMT-related genes (Al-Ansari and Aboussekhra, 2014; Chang et al., 2017; Fehrholz et al., 2014). Regardless of its mechanism in the late administration, the results in this study should provide information of health awareness for pregnant women who are intensive intakers of caffeine-enriched beverage daily. It should be noted that the dose that we used in this study (2 mg/ D3 embryos (20 g in weight) or equivalent to 100 mg/kg) is relative lower than LD50 dose in humans (150-200 mg/kg), the dose which already posted many severe effects in the ventricle. Consuming high amount of caffeine daily would encounter a high risk of exposing pejorative effect of caffeine, of which its sensitivity towards receptor may depend on an individual person that exposes to caffeine.

ACKNOWLEDGEMENTS

This work was supported by joint funding between Thailand Research Fund and Mahidol University (TRF, Grant # RSA5980029 to SA), Faculty of Science, Mahidol University (to WW) and Walailak University (to NC). We thank Mrs. Somrudee Attachit and scientists at the Center of Nanoimaging (CNI) and the Central Instrument Facility (CIF), Faculty of Science, Mahidol University, for providing instrumental support throughout this work. We would like to thank Prof. Wouter H. Lamers, Development of Anatomy & Embryology, Maastricht University, Maastricht, the Netherlands for his valuable comments and manuscript proofreading.

REFERENCES

AANHAANEN WT, MOMMERSTEEG MT, NORDEN J, WAKKER V, de GIER-de VRIES C, ANDERSON RH, KISPERT A, MOORMAN AF, CHRISTOFFELS V (2010) Developmental origin, growth, and three-dimensional architecture of the atrioventricular conduction axis of the mouse heart. Circ Res, 107: 728-736.

Al-ANSARI MM, ABOUSSEKHRA A (2014) Caffeine mediates sustained inactivation of breast cancer-associated myofibroblasts via up-regulation of tumor suppressor genes. PLoS One, 9: e90907.

BAN Q, LIU X, HUI W, CHEN D, ZHAO Z, JIA B (2013) Comparative analysis of Nkx2- 5/GATA4/TBX5 expression in chicken, quail and chicken-quail hybrids during the early stage of cardiac development in embryos. AJAS, 26: 476-482.

BRAND T (2003) Heart development: molecular insights into cardiac specification and early morphogenesis. Dev Biol, 258: 1-19.

BRUNEAU BG (2002) Transcriptional regulation of vertebrate cardiac morphogenesis. Circ Res, 90: 509-519.

BRUNEAU BG (2013) Signaling and transcriptional networks in heart development and regeneration. CSH Perspect Biol, 5: a008292.

BUSCARIOLLO DL, BREUER GA, WENDLER CC, RIVKEES SA (2011) Caffeine acts via A1 adenosine receptors to disrupt embryonic cardiac function. PLoS One, 6: e28296.

BUTLER H, JUURLINK BHJ (1987) An Atlas for Staging Mammalian and Chick Embryos. Boca Ratón, Florida: CRC Press.

CAI C, LIANG X, SHI Y, CHU P, PFAFF S, CHEN J, EVANS S (2003) Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell, 5: 877-889.

CARLSON BM (1981) Patten's Foundations of Embryology (4 ed.). Shepherdstown, WV, U.S.A.: BookDepart.

CAVALCANTI JS, DUARTE SM (2008). Estudo morfométrico do coração fetal: sua utilização como parâmetro para análise ecocardiográfica. J Radiologia Brasileira, 41: 99-101.

CHANG YL, HSU YJ, CHEN Y, WANG YW, HUANG SM (2017) Theophylline exhibits anti-cancer activity via suppressing SRSF3 in cervical and breast cancer cell lines. Oncotarget, 8: 101461-101474.

CHEN L, BELL EM, BROWNE ML, DRUSCHEL CM, ROMITTI PA (2014) Exploring maternal patterns of dietary caffeine consumption before conception and during pregnancy. Matern Child Health J, 18: 2446-2455.

CHEN Y, CHOU WC, DING YM, WU YC (2014) Caffeine inhibits migration in glioma cells through the ROCK-FAK pathway. Cell Physiol Biochem, 33: 1888-1898.

COMBS MD, YUTZEY KE (2009) Heart valve development: regulatory networks in development and disease. Circ Res, 105: 408-421.

CREAZZO T, GODT R, LEATHERBURRY LJ, CONWAY S, KIRBY M (1998) Role of cardiac neural crest cells in cardiovascular development. Annu Rev Physiol, 60: 267-286.

De BAKKER BS, DE JONG KH, HAGOORT J, de BREE K, BESSELINK CT, de KANTER FEC, VELDHUIS T, BAIS B, SCHILDMEIJER R, RUIJTER JM, OOSTRA RJ, CHRISTOFFELS VM, MOORMAN AFM (2016) An interactive three-dimensional digital atlas and quantitative database of human development. Science, 354: aag0053.

DUNN KK, PALECEK SP (2018) Engineering scalable manufacturing of high-quality stem cell-derived cardiomyocytes for cardiac tissue repair. Front Med, 5: 110.

ECHEVERRI D, MONTES FR, CABRERA M, GALÁN A, PRIETO A (2010) Caffeine's vascular mechanisms of action. Int J Vasc Med, 2010: 834060.

ENGELMANN GL, VITULLO JC, GERRITY RG (1987) Morphometric analysis of cardiac hypertrophy during development, maturation, and senescence in spontaneously hypertensive rats. Circ Res, 60: 487-494.

EVANS SM, YELON D, CONLON FL, KIRBY ML (2010) Myocardial lineage development. Circ Res, 107: 1428-1444.

FEHRHOLZ M, SPEER CP, KUNZMANN S (2014) Caffeine and rolipram affect Smad signalling and TGF-beta1 stimulated CTGF and transgelin expression in lung epithelial cells. PLoS One, 9: e97357.

FUSTER V, ALEXANDER RW, O'ROURKE RA (2011) Hurst's the Heart (13 ed.). New York: McGraw-Hill.

GALDOS FX, GUO Y, SHARON LP, VanDUSEN NJ, WU SM, WILLIAM TP (2017) Cardiac regeneration: lessons from development. Circ Res, 120: 941-959.

HOOGAAS W, BARNETT P, MOOMAN AFM, CHRISTOFFELS V (2007) T-box factors determine cardiac design. Cell Mol Life Sci, 64: 646-660.

HOPWOOD N (2002) Embryos in wax: models from the Aiegler studio. Cambridge: Whipple Museum of the History of Science: University of Cambridge.

HUTSON MR, KIRBY ML (2007) Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. Semin Cell Dev Biol, 18: 101-110.

KELLY RG (2012) The second heart field. Curr Top Dev Biol, 100: 33-65.

KELLY RG, BROWN N, BUCKINGHAM M (2001) The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev Cell, 1: 435-440.

KIM GE, KASS DA (2017) Cardiac phosphodiesterases and their modulation for treating heart disease. Handbook Exp. Pharmacol, 243: 249-269.

KOBAYASHI T, NISHIDA A, KUROKAWA A, ARIYUKI F (1995) Cardiovascular malformations induced by caffeine and phenobarbital in chick embryos. AATEX, 3: 17-27.

KOLANOWSKI TJ, ANTOS CL, GUAN K (2017) Making human cardiomyocytes up to date: Derivation, maturation state and perspectives. Int J Cardiol, 241: 379-386.

KRUEPUNGA N, HIKSPOORS J, MEKONEN HK, MOMMEN GMC, MEEMON K, WEERACHATYANUKUL W, ASUVAPONGPATANA S, ELEONORE KŐHLER S, LAMERS WH (2018) The development of the cloaca in the human embryo. J Anat, 233: 724-739.

LEE H, NAGELE RG, PIETROLUNGO JF (1982) Toxic and teratologic effects of caffeine on explanted early

chick embryos. Teratology, 25: 19-25.

LI J, ROBERTSON DR, LEMANSKI LF (1994) Morphometric analysis of cultured normal and cardiomyopathic hamster heart cells after immunofluorescent staining for tubulin and α-actinin. Acta Histochem, 96: 33-42.

LIU CY, LIN HH, TANG MJ, WANG YK (2015) Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget, 6: 15966-15983.

LUIDER TM, BRAVENBOER N, MEIJERS C, van der KAMP AW, TIBBOEL D, POELMANN RE (1993) The distribution and characterization of HNK-1 antigens in the developing avian heart. Anat Embryol, 188: 307-316.

O'RAHILLY R, MULLER F (2010) Developmental stages in human embryos: revised and new measurements. Cells Tissues Organs, 192: 73-84.

PAIGE SL, PLONOWSKA K, XU A, WU SM (2015) Molecular regulation of cardiomyocyte differentiation. Circ Res, 116: 341-353.

RIVKEES SA, WENDLER CC (2012) Regulation of cardiovascular development by adenosine and adenosine -mediated embryo protection. Arterioscl Throm Vas, 32: 851-855.

RIVKEES SA, WENDLER CC (2017) Long-term consequences of disrupting adenosine signaling during embryonic development. Mol Aspects Med, 55: 110-117.

RUNYAN R, HEIMARK R, CAMENISCH T (2013) Epithelial-mesenchymal transformation in the embryonic heart. https://www.ncbi.nlm.nih.gov/books/NBK6272/

SAMSA LA, YANG B, LIU J (2013) Embryonic cardiac chamber maturation: Trabeculation, conduction, and cardiomyocyte proliferation. Am J Med Genet Part C Semin Med Genet, 163C: 157-168.

von GISE A, PU WT (2012) Endocardial and epicardial epithelial to mesenchymal transitions in heart development and disease. Circ Res, 110: 1628-1645.

WENDLER CC, BUSOVSKY-McNEAL M, GHATPANDE S, KALINOWSKI A, RUSSELL KS, RIVKEES SA (2009) Embryonic caffeine exposure induces adverse effects in adulthood. FASEB J, 23: 1272-1278.

YELON D (2001) Cardiac patterning and morphogenesis in zebrafish. Dev Dyn, 222: 552-563.

ZAFFRAN S, FRASCH M (2002) Early signals in cardiac development. Circ Res, 91: 457-469.