Enhance Smooth Muscle Cells Apoptosis Associated With Pulmonary Arterial Remodeling in Dogs Affected With Pulmonary Hypertension Secondary to Degenerative Mitral Valve Disease

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Research Article

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Enhance smooth muscle cells apoptosis associated with pulmonary arterial remodeling in dogs affected with pulmonary hypertension secondary to degenerative mitral valve disease

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Abstract

Background: Degenerative mitral valve disease (DMVD) is the most common cause of pulmonary hypertension (PH) in dogs. Medial thickening of the pulmonary artery is a major histopathological change in PH. A decrease in apoptosis of pulmonary arterial smooth muscle cells (SMCs) may be the cause of medial thickening. This study aimed to demonstrate the expression of apoptosis molecules in the pulmonary artery of dogs affected with PH secondary to DMVD (DMVD+PH) compared to DMVD without PH (DMVD) and healthy dogs (control). Lung samples were collected from three groups including control (n=5), DMVD (n=7) and DMVD+PH (n=7) groups. Masson trichrome and apoptotic proteins including Bax, Bcl2 and caspase-3 and -8, were stained.

Results: The medial thickness in the DMVD and DMVD+PH groups was greater than in the control group and it was greatest in the DMVD+PH group. Bax, Bcl2 and caspase-3 and -8 were expressed mainly in the medial layer of the pulmonary artery. The percentages of Bax and caspase-3 and -8 positive cells were higher in the DMVD group compared to the DMVD+PH group, whereas the percentage of Bcl2-positive cells was increased in the DMVD and DMVD+PH groups. These findings suggested that apoptosis of pulmonary arterial SMCs occurred mainly in the DMVD group and decreased dramatically in the DMVD+PH group.

Conclusions: An increase in the medial thickness in dogs affected with PH secondary to DMVD may occur due to a decrease in apoptosis of pulmonary arterial SMCs.

Keywords: Apoptosis, Degenerative mitral valve disease, Medial thickening, Pulmonary hypertension, Smooth muscle cells

Background

Pulmonary hypertension (PH) involves a hemodynamic and pathophysiological change in pressure within the pulmonary vasculature. It has been defined as an abnormal increase in pulmonary arterial systolic pressure (PAP). In veterinary medicine, dogs with clinical signs of PH, such

as dyspnoea and syncope and an estimated PAP of > 46 mmHg measured by echocardiography, are defined with PH (1).

Degenerative mitral valve disease (DMVD) is a left-sided heart disease frequently found in small-sized breed dogs (2), and it is the most common cause of PH in dogs (1, 3). As the disease progresses, the degenerative valve cannot close properly during systole causing mitral valve regurgitation and volume overload in the left atrium. In the advanced stage, some dogs may suffer from left-sided heart failure (4) and/or develop complications such as PH and right-sided heart failure (3, 5-7). DMVD dogs with clinical signs such as syncope, respiratory distress and exercise intolerance are suggestive of PH (1, 7). While right heart catheterisation is a gold-standard method for diagnosing PH in human patients, the suggestive clinical signs of PH and echocardiographic parameters, including estimated PAP and echocardiographic signs of PH involving ventricles, pulmonary artery, right atrium and caudal vena cava, have been used to diagnose PH in dogs (1, 3, 8, 9).

Pulmonary arterial remodelling is the histopathological changes in the pulmonary artery found in human patients affected with PH and in PH-induced animal models. The histopathological changes affect all layers of the pulmonary arterial wall, including the intimal, medial and adventitial layers (3, 10). The deposition of fibroblasts and connective tissues in the intimal and adventitial layers are referred to as intimal and adventitial fibrosis, respectively (11). Medial remodelling causing medial thickening is a hallmark structural change in the pulmonary artery found in patients affected with PH secondary to several causes, including chronic obstructive pulmonary disease (12), idiopathic PH (13) and left-sided heart failure (14, 15) and in PH-induced animal models (16-20). The aetiology of medial thickening remains unknown. The homeostasis of pulmonary arterial SMC proliferation and apoptosis is essential for maintaining the normal structure of the pulmonary artery, and the imbalance of cell death and proliferation in SMCs may play a role in medial thickening. The decrease in apoptosis of pulmonary arterial SMCs has been suggested as a possible cause of the increased number of SMCs and medial thickness in the pulmonary artery (21).

Apoptosis is a complex mechanism that involves two main pathways: intrinsic and extrinsic pathways. The extrinsic pathway is initiated by cells, no longer needed, receiving an external signal, which then transmits from the cell surface to intracellular signal pathways, resulting in activation of caspase-8 and then promoting activation of effector caspase-3. The intrinsic pathway is triggered by stimuli that cause stress or damage to the cell. Intracellular signals cause a change in the mitochondrial membrane leading to the release of cytochrome c to the cytoplasm, forming apoptosomes, cleaving and activating effector caspase-3 (22, 23). Therefore, caspase-3 is an essential effector caspase responsible for extrinsic and intrinsic pathways and causing a breakdown of DNA and chromatin condensation (22, 24). Apart from caspase proteins, cytoplasmic proteins called Bcl2 family proteins, are also associated with the apoptotic pathway. The Bcl2 family proteins can be categorised into two main groups, manifesting pro-apoptotic and anti-apoptotic responses, including pro-apoptotic Bcl2 associated protein (Bax) and anti-apoptotic B-cell lymphoma 2 protein (Bcl2), respectively (22). The ratio of Bax to Bcl2 appears to be the prognostic factor for determining the susceptibility of cells to apoptosis (24).

Several studies have suggested that a decrease in apoptosis of pulmonary arterial SMCs is associated with increased medial thickness in human patients and in animal models with PH (25-31). Moreover, induction or stimulation of pulmonary arterial SMCs apoptosis can reverse PAP, restore the pulmonary artery to its normal structure or prevent medial thickening progression (23, 32). However, there is no study investigating the expression of proteins related to apoptosis in the pulmonary artery of dogs affected with PH secondary to DMVD. Therefore, this study aimed to demonstrate the expression of molecules in apoptosis pathways in the pulmonary artery of dogs affected with PH secondary to DMVD compared to DMVD without PH and healthy control dogs.

Results

Clinical characteristics of dogs

The control group consisted of 5 dogs including 3 males (M=1, Mc=2) and 2 females (F=1, Fs=1) with breeds of Shih Tzu (n=3), Mixed and Pomeranian. The DMVD group consisted of 7 dogs including 3 males (M=1, Mc=2) and 4 females (F=2, Fs=2) with breeds of Poodle (n=4), Shih Tzu (n=2) and Pomeranian. The DMVD+PH group consisted of 7 dogs including 4 males (M=2, Mc=2) and 3 females (F=2, Fs=1) with breeds of Poodle (n=2), Chihuahua (n=2), Shih Tzu, Pomeranian and Schnauzer. Age and weight of dogs among the control (11.2±3.63 year; 5.59±0.38 kg), DMVD (13.71±1.80 year; 4.91±1.49 kg) and DMVD+PH (13.86±1.57 year; 6.25±2.99 kg) groups were not significantly different (p=0.136, 0.486). Sex among groups were approximately the same. Almost all of dogs in the DMVD and DMVD+PH group were died from cardiovascular failure. Only one dogs in the DMVD+PH group was euthanized due to unresponsive to the standard therapy. Dogs in the control group were died secondary to postoperative complication and car accident. The average estimated PAP of dogs in the DMVD+PH group was 75.77 ± 28.79 mmHg (range 49.51-116 mmHg).

Pulmonary arterial remodeling

The main histopathological changes of lung in the DMVD and DMVD+PH groups were congestion and pulmonary edema. Masson trichrome staining showed interstitial alveolar fibrosis in some dogs in the DMVD+PH group (2 of 7 dogs). Microscopic examination of pulmonary arteries showed thin medial layer in the control group, whereas multiple layers of SMCs in the medial layer was presented in all dogs in the DMVD and DMVD+PH groups (Fig.1).

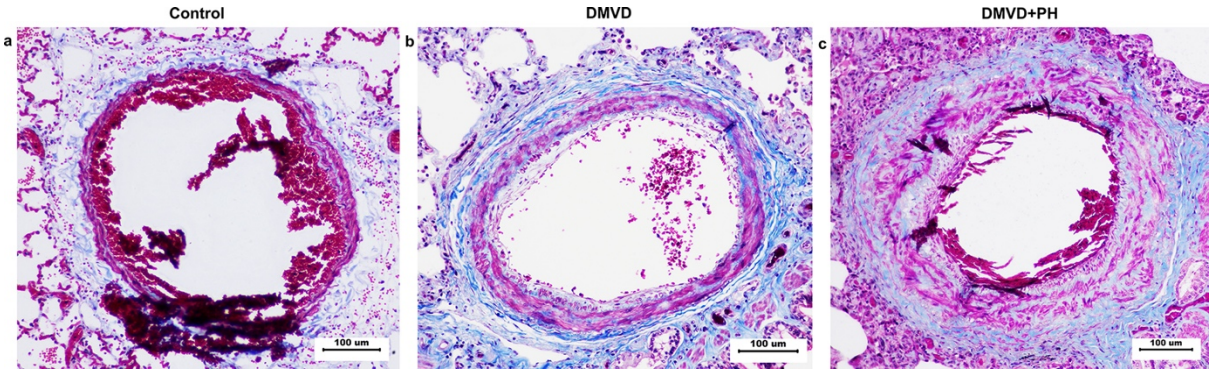


Figure 1: The histopathology of the pulmonary artery

Masson's trichrome staining revealed normal thin medial layer in the control group (a) and increased medial thickness in the degenerative mitral valve disease (DMVD) (b) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) (c) groups. Red color are staining of SMCs; Blue color are staining of collagen fibers (Masson's trichrome stain, 100x magnification).

The percentage of medial thickness (%MT) was significantly increased in the DMVD and DMVD+PH groups compared to the control group ($p<0.0001$). Moreover, dogs in the DMVD+PH group had %MT greater than those in the DMVD group ($p<0.0001$) (Table1, Fig.2). The average number in the DMVD and DMVD+PH groups were significantly increased compared to the control group and it was highest in the DMVD+PH group (Table1, Fig.2). The percentage of medial thickness showed strong correlation with the average number of SMCs ($r=0.894$, $p<0.0001$).

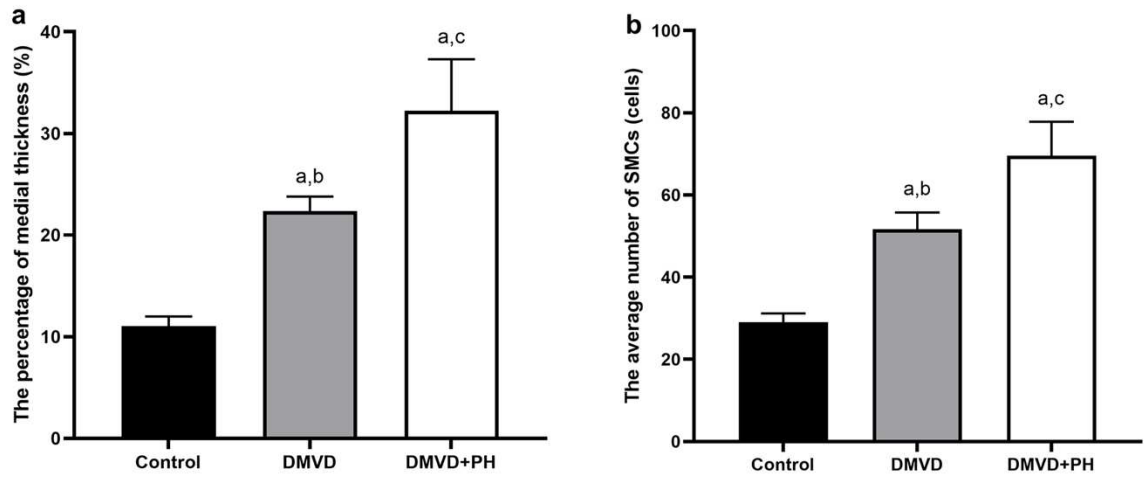


Figure 2: The percentage of medial thickness and the average number of pulmonary arterial SMCs per vessel.

The graphs show the percentage of medial thickness (%MT) (a) and the average number of pulmonary arterial SMCs per vessel (b) of the control, degenerative mitral valve disease (DMVD) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups. Data are expressed as mean and standard deviation (SD) (bars). An increase in %MT in the DMVD and DMVD+PH groups conforms to an increase in the average number of pulmonary arterial SMCs.

[a] Indicate statistically significant difference at $p < 0.05$ compared to the control group.

[b,c] Indicate statistically significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.

Table 1 The percentage of medial thickness and the average number of pulmonary arterial SMCs per vessel

Parameter	Control (n=5)	DMVD (n=7)	DMVD+PH (n=7)	p-value
%MT	11.04 ± 0.95	22.36 ± 1.44[a, b]	32.25 ± 5.06[a, c]	[a] <0.0001 [b, c] <0.0001
SMCs (cells)	29.04 ± 2.15	51.65 ± 4.11[a, b]	69.53 ± 8.33[a,c]	[a] <0.0001 [b, c] <0.0001

Data are expressed as mean ± standard deviation (SD).

The significant difference was assessed by one-way ANOVA at $p < 0.05$.

[a] Indicate significant difference at $p < 0.05$ compared to the control group.

[b, c] Indicate significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.

Expression of proteins associated with apoptosis pathways

Immunohistochemistry was performed to localize proteins associated with apoptosis pathways including Bax (pro-apoptotic protein), Bcl2 (anti-apoptotic protein), caspase-3 and caspase-8 (caspase proteins). The results showed that Bax, Bcl2, caspase-3 and caspase-8 were mainly expressed in medial layers of pulmonary arteries (Fig.3,4).

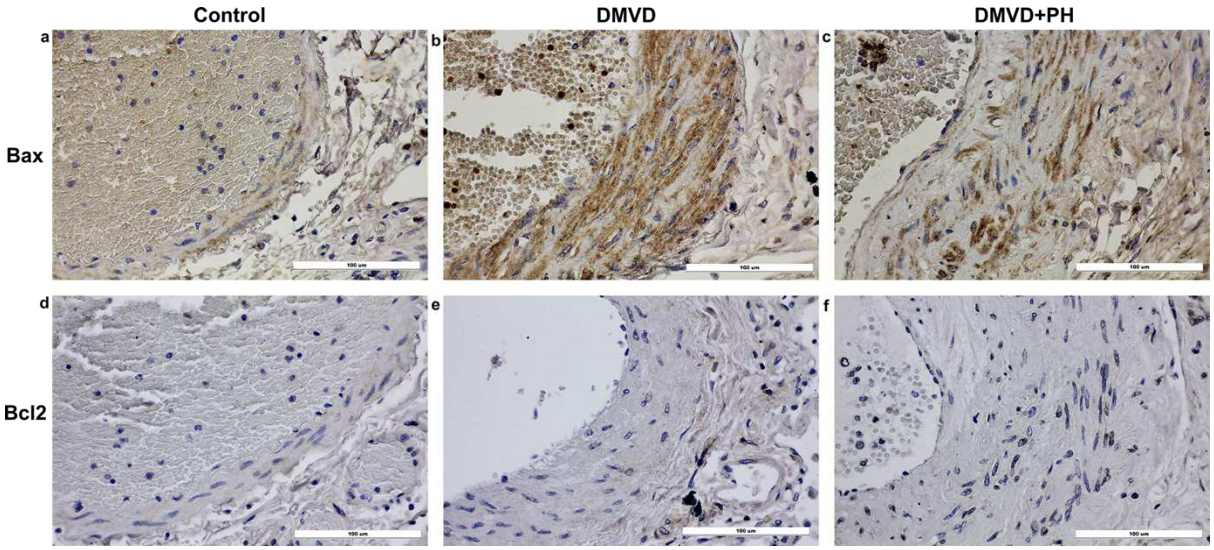


Figure 3: Immunohistochemical expression of Bax and Bcl2 in pulmonary arteries.

The images shows the expression of Bax and Bcl2 in the pulmonary arteries of the control (a, d), degenerative mitral valve disease (DMVD) (b, e) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups (c, f). These proteins were mainly expressed in SMCs located at medial layer of pulmonary artery. Bax protein was stained in all groups, whereas Bcl2 protein was not stained in the control group. The intracytoplasmic staining of Bax and intranuclear staining of Bcl2 in pulmonary arterial SMCs presented as brown color (Labeled streptavidin-biotin, Immunohistochemistry, Mayer's Hematoxylin counterstained, 400x magnification).

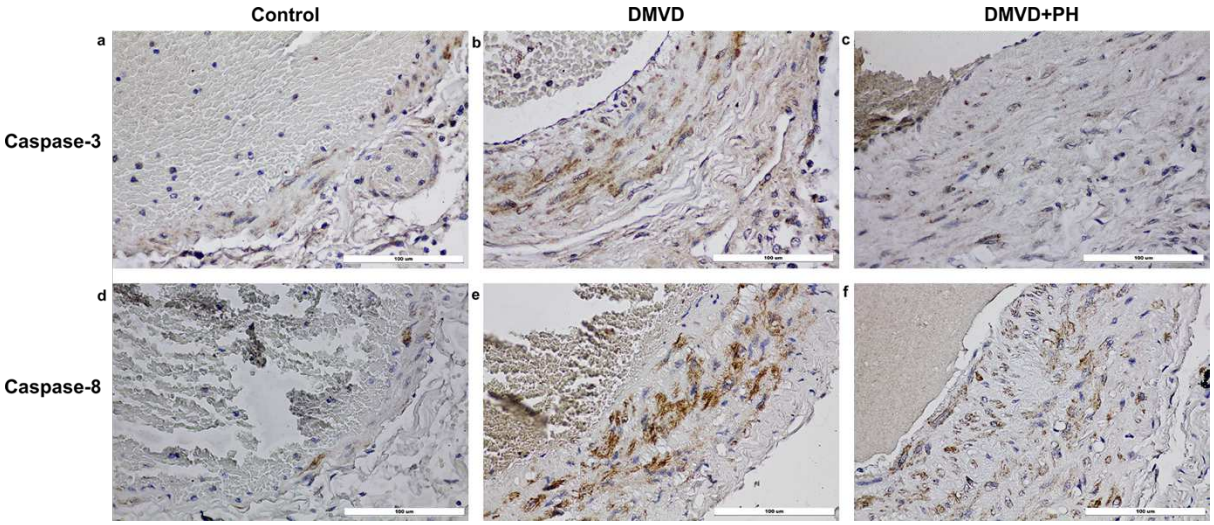
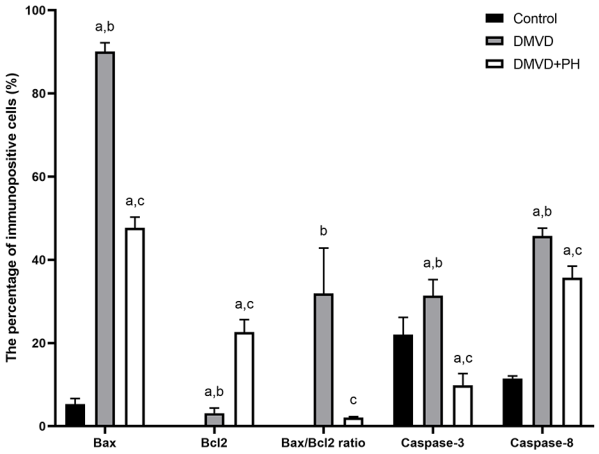


Figure 4: Immunohistochemical expression of caspase-3 and -8 in pulmonary arteries.

The images show the expression of caspase-3 and -8 in the pulmonary arteries of the control (a, d), degenerative mitral valve disease (DMVD) (b, e) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups (c, f). The caspase-3 and -8 expressions were mainly found in SMCs located at the medial layer of the pulmonary artery in all 3 groups. The intracytoplasmic and intranuclear staining of caspase-3 and intracytoplasmic staining of caspase-8 were in brown color. (Labeled streptavidin-biotin, Immunohistochemistry, Mayer's Hematoxylin counterstained, 400x magnification).

Intracytoplasmic staining of Bax was detected in SMCs located in the medial layer of dogs in the control, DMVD and DMVD+PH groups (Fig.3). The percentage of Bax positive cells in the DMVD and DMVD+PH groups were higher than the control group ($p<0.0001$). However, it was decreased in the DMVD+PH group compared to the DMVD group ($p<0.0001$) (Table2, Fig.5). Intranuclear Bcl2 expression was found only in DMVD dogs with and without PH but not in control dogs (Fig.3). Contrary to Bax, Bcl2 expression was very low in the DMVD group and significantly increased in the DMVD+PH group ($p<0.0001$) (Table2, Fig.5). The Bax to Bcl2 ratio was high in the DMVD group and dramatically reduced in the DMVD+PH group ($p<0.0001$) (Table2, Fig.5).



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198 **Figure 5: The percentage of Bax, Bcl2, and caspase-3 and -8 positive cells and Bax/Bcl2 ratio**

199 The graph show the percentage of Bax, Bcl2, and caspase-3 and -8 positive cells and Bax/Bcl2 ratio of the
200 control, degenerative mitral valve disease (DMVD) and degenerative mitral valve disease with pulmonary
201 hypertension (DMVD+PH) groups. Data are expressed as mean and standard deviation (SD) (bars). The
202 percentage of Bax and caspase-3 and -8 positive cells as well as Bax/Bcl2 ratio were highest in the DMVD
203 group, opposite to the percentage of Bcl2 positive cells that was highest in the DMVD+PH group.

204 [a] Indicate statistically significant difference at $p < 0.05$ compared to the control group.

205 [b,c] Indicate statistically significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.

206

207 As for downstream signaling proteins, caspase-3 and caspase-8 proteins were stained in
208 pulmonary arterial SMCs of dogs in all 3 groups. Caspase-3 was expressed in the nucleus and
209 cytoplasm, whereas caspase-8 was expressed only in the cytoplasm of pulmonary arterial SMCs
210 (Fig.4). The percentage of caspase-3 and caspase-8 positive cells was highest in the DMVD group
211 compared to the control and DMVD+PH groups. Interestingly, %caspase-3 positive cells was very low
212 in the DMVD+PH groups and lower than the control group. However, %caspase-8 positive cells in the
213 DMVD+PH group was higher than the control group (Table2, Fig.5).

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Table 2 The percentage of Bax, Bcl2 and caspase-3 and -8 positive cells and Bax/Bcl2 ratio

Parameter	Control (n=5)	DMVD (n=7)	DMVD+PH (n=7)	p-value
%Bax	5.32 ± 1.36	90.13 ± 2.08[a, b]	47.69 ± 2.61[a, c]	[a] <0.0001 [b, c] <0.0001
%Bcl2	0	3.16 ± 1.24[a, b]	22.65 ± 3.00[a, c]	[a] <0.0001 [b, c] <0.0001
%Caspase-3	22.05 ± 4.13	31.42 ± 3.86[a, b]	9.88 ± 2.81[a,c]	[a] <0.0001 [b, c] <0.0001
%Caspase-8	11.51 ± 0.61	45.78 ± 1.85[a, b]	35.73 ± 2.77[a,c]	[a] <0.0001 [b, c] <0.0001
Bax/Bcl2 ratio	-	31.97 ± 10.86[b]	2.12 ± 0.19[c]	[b,c] <0.0001

Data are expressed as mean ± standard deviation (SD).

The significant difference of %Bax, %Bcl2, %caspase-3 and %caspase-8 were assessed by one-way ANOVA at $p < 0.05$.

The significant difference of Bax/Bcl2 ratio was assessed by independent t-test at $p < 0.05$.

[a] Indicate significant difference at $p < 0.05$ compared to the control group.

[b, c] Indicate significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.

The percentage of medial thickness and the average number of pulmonary arterial SMCs per area showed a similar correlation pattern with apoptosis associated proteins. The percentage of Bcl2 positive cells was positively correlated with %MT ($r=0.829$, $p<0.0001$) and the average number of pulmonary arterial SMCs per area ($r=0.867$, $p<0.0001$). The percentage of caspase-3 positive cells was negatively correlated with %MT ($r=-0.498$, $p=0.03$) and the average number of pulmonary arterial SMCs per area ($r=-0.466$, $p=0.044$).

Discussion

The aim of this study was to determine whether or not the apoptosis resistance of pulmonary arterial SMCs was associated with medial thickening in the dogs affected with PH secondary to DMVD or not. The results of the study suggested that apoptosis of pulmonary arterial SMCs was dramatically decreased in the DMVD+PH group.

The main histopathological changes in the lungs in DMVD and DMVD+PH groups were pulmonary oedema and congestion, which is consistent with the results from a previous study that examined pulmonary histopathology from a surgical biopsy of the lungs of DMVD dogs (33). Interestingly, some dogs in the DMVD+PH group showed alveolar fibrosis, which is characterised by increased collagen fibres and reactive fibroblasts in the alveolar septum and usually occurs secondary to lower respiratory tract injury (34-36). Chronic increased hydrostatic forces in the pulmonary capillary bed in dogs with left-sided heart failure dogs can cause damage to the alveolar and interstitial structure, and the healing process forms a scar tissue and fibrosis (33).

Medial thickening is a hallmark structural change in the pulmonary artery found in human patients with PH secondary to several causes (12-14, 37) as well as in PH- induced animal models (16-18). The present study revealed that %MT was increased in the DMVD and DMVD+PH groups compared to the control group. Similar findings have been reported in human patients affected with left-sided heart failure both with and without PH (14). Interestingly, %MT in the DMVD group was higher than in the DMVD+PH group. These findings suggest that increased medial thickness could occur even in DMVD dogs without PH and progress continually in DMVD dogs with PH.

The cause of medial thickening in subjects affected with PH is not yet completely understood (38, 39). An increase in the number of pulmonary arterial SMCs may be one of the causes of medial thickening. The present study showed that the total number of pulmonary arterial SMCs was significantly increased in the DMVD and DMVD+PH groups compared to the control group, was significantly higher in the DMVD+PH group than those in the DMVD group. In addition, a positive correlation was found between the total number of pulmonary arterial SMCs and %MT, suggesting

that an increase in the number of pulmonary arterial SMCs probably contributes to medial thickening in DMVD dogs both with and without PH.

An increase in the number of pulmonary artery SMCs may occur secondary to the lack of apoptosis (21, 23) (20, 29, 31, 40-42). Apoptosis is a mechanism that maintains the homeostasis of tissues by eliminating unwanted cells. Several proteins are involved in apoptotic pathways, including Bax, Bcl2 and caspase-3 and -8. Bcl2 blocks apoptosis, whereas Bax promotes it. Caspase-3 and -8 are important caspases associated with intrinsic and extrinsic apoptotic pathways (24). Several studies have determined the expression of these proteins to evaluate cell apoptosis in several diseases and conditions, including PH (24-30, 43, 44). To the authors' knowledge, this study is the first to evaluates the expression of proteins associated with apoptosis pathways in the pulmonary arteries of dogs affected with PH secondary to DMVD. The results of the study showed that Bax, Bcl2 and caspase-3 and -8 are expressed in SMCs in the medial layer of pulmonary arteries in a similar way to that found in humans (29) and animal models (25-28, 30, 44) with PH.

Bax, a pro-apoptotic protein, was found in all three groups, whereas Bcl-2, an anti-apoptotic protein, was not found in the control group. The percentage of Bax positive cells (%Bax) was very low in the control group, suggesting that an apoptotic process could be found in normal conditions to limit the number of cells in the pulmonary arterial wall (23). % Bax was increased markedly in the DMVD dogs compared to the normal dogs; however, it was decreased in the DMVD+PH group compared to that in the DMVD group, indicating that apoptosis of pulmonary arterial SMCs occurred mainly in the DMVD group and was reduced in the DMVD+PH group. This finding was in agreement with previous studies in animal models that demonstrated a decrease or absence of Bax expression in the pulmonary arteries of subjects with PH compared to the controls (25-28).

The expression of Bcl2, an anti-apoptotic protein, was higher in the DMVD+PH group compared to the other groups. The percentage of Bcl2 positive cells (%Bcl2) was very low in the DMVD group and significantly increased in the DMVD+PH group, which is consistent with studies in animal models (25-28). The results suggest that apoptosis resistance occurred mainly in the

pulmonary arterial SMCs of the DMVD dogs with PH. The Bax to Bcl2 ratio can be used to estimate the apoptotic susceptibility of cells: cells with high Bax to Bcl2 ratio are more prone to apoptosis (24, 30). This ratio was decreased in the DMVD+PH group compared to the DMVD group, indicating that the susceptibility to apoptosis was decreased in the DMVD+PH group compared to the DMVD group.

The expression of caspase-3 and -8, pro-apoptotic proteins, was correlated with the apoptotic rate. A higher expression of caspase-3 and-8 suggests more apoptosis (45). Caspase-3 is an effector protein related to the extrinsic and intrinsic pathways of apoptosis, whereas caspase-8 is an initiator protein responsible for an extrinsic apoptotic pathway of apoptosis (22, 24). Although caspase-3 expression has been used to evaluate apoptosis in several diseases and conditions including PH, no study has investigated the expression of caspase-8 in subjects suffering from PH. The present study selected caspase-3 and -8 for this study to determine whether intrinsic or extrinsic pathways or both are involved in apoptosis in DMVD dogs with PH. The expression of caspase-3 and-8 was found in the medial layer of pulmonary arteries in the three groups, but The percentage of caspase-3 and -8 positive cells (%caspase-3, %caspase-8) were highest in the DMVD group compared to the control and the DMVD+PH groups. Taken together with the results for Bax and Bcl2, it appeared that apoptosis of pulmonary arterial SMCs occurred mainly in the DMVD group and was dramatically decreased in the DMVD+PH group. Interestingly, %caspase-3 in the DMVD+PH group was lower than in the control group, similar to the study in PH-induced animal models (25, 27), whereas %caspase-8 in the DMVD+PH group was higher than that in the control group. Moreover, %MT and the number of pulmonary artery SMCs did not correlate with %caspase-8 was negatively correlated with %caspase-3. These findings suggest that the major pathway involved in the apoptosis resistance in DMVD dogs with PH may be an intrinsic, not an extrinsic, pathway. Despite apoptosis occurring mainly in DMVD dogs, they also presented with medial thickening. Therefore, medial thickening in the DMVD dogs may not be caused by a decrease in apoptosis but may be caused by an increase in pulmonary arterial SMCs proliferation.

The present study has limitations that need to be addressed. A Terminal deoxynucleotidyl transferase-mediated deoxyuridine tripjosphate nick-end labelling (TUNEL) assay was not performed to detect DNA strand breaks in apoptotic cells. However, the immunohistochemical staining of Bax, Bcl2 and caspase-3 and -8 has been widely used to evaluate apoptosis in several diseases and conditions, including PH in human patients and animal models (24-31), and the detection of activating caspase-3 is reflects the induction of cell death by apoptosis. Therefore, the expression of these proteins is sufficient for evaluating apoptosis in the cells. In addition, commercial antibodies against dogs of canine Bax, Bcl2 and caspase-3 and-8 are not available. So, this study used antibodies against human Bax, Bcl2 and caspase-3 and-8 instead of dogs. According to the amino acid sequence of these proteins provided by Uniprot, canine Bax, Bcl2 and caspase-3 and -8 are highly homologous (>80%) to those of humans. Therefore, these antibodies were probably suitable for use in this study.

Conclusion

In conclusion, an increase in the medial thickness of the pulmonary artery in DMVD dogs with PH may be partly because of a decrease in apoptosis or the resistance to apoptosis of pulmonary arterial SMCs. This study leads to a better understanding of the pathogenesis of PH in DMVD dogs and the results of this study could have a considerable impact in the future on apoptosis-based therapy to treat or prevent pulmonary arterial remodeling in PH dogs secondary to DMVD in the future.

Methods

Tissue samples

Lung samples were obtained from 19 carcasses of ageing small-sized breed dogs presenting at Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University for necropsy. Dogs with pulmonary diseases, heartworm infection, systemic hypertension, neoplasia or other systemic diseases such as kidney and liver diseases determined by antemortem diagnostic

records and gross pathology were excluded from this study. All samples were divided into three groups, including control (n=5), degenerative mitral valve disease (DMVD) (n=7) and DMVD with pulmonary hypertension (DMVD+PH) (n=7) based on antemortem diagnostic history and mitral valve thickness evaluated at necropsy. To be included to the control group, dogs had to have normal blood profile values and a mitral valve thickness of < 2 mm measured by a Vernier caliper (46, 47). DMVD dogs were dogs those that had been diagnosed as DMVD stage C (vertebral heart score more than 10.5 with current or past radiographic signs of pulmonary edema, left atrial and left ventricle enlargement [LA/Ao ≥ 1.6 (Swedish method) and left ventricular internal diameter during diastole normalized with Allometric scale method (LVIDdN) ≥ 1.7 assessed by echocardiography before died) (48) and had a mitral valve thickness of > 2 mm at necropsy. DMVD dogs without PH were included in the DMVD group. To be included into DMVD+PH group, DMVD dogs had to have an intermediate-to-high probability of PH secondary to DMVD evaluated by estimated PAP and anatomic structural changes assessed by echocardiography (1, 8).

Immunohistochemistry

Lung tissues were collected from the peripheral regions of right caudal lung lobes and fixed in 10% formalin for 24 hours and embedded in paraffin blocks. Four-micrometre thick lung tissue sections were stained with Masson trichrome for examination of medial thickness. The immunohistochemical staining was performed to evaluate proteins associated with apoptosis pathways including Bax (pro-apoptosis protein), Bcl2 (anti-apoptosis protein) and caspase-3 and caspase-8 following the protocols described previously (24, 26-30, 43, 44, 49). Briefly, lung tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, pretreated with citrate buffer (0.01M, pH 6.0), blocked endogenous peroxidase activity and nonspecific binding by 3% hydrogen peroxide and 1% bovine serum albumin, respectively. Lung tissue sections were then incubated with primary antibodies including polyclonal rabbit anti-Bax (1:100) (A3533, Dako, Glostrup, Denmark), monoclonal mouse anti-Bcl2 (1:100) (NCL-bcl-2-486, Leica Biosystems, Newcastle, UK), rabbit polyclonal anti-caspase-3 (1:100) and caspase-8 (1:100) (ab4051, ab4052, Abcam, Cambridge, UK)

overnight. After that lung tissue sections were incubated with horse radish peroxidase-labeled polymer conjugated with secondary antibody followed by 3,3'-diaminobenzidine tetrahydrochloride for visualizing peroxidase activity (Envision Detection System, Peroxidase/DAB, Rabbit/Mouse, Dako, Hamburg, Germany). Finally, lung tissue sections were counterstained with Mayer's hematoxylin.

Ten pulmonary arteries with external diameter of approximately 300 μm examined by light microscopy were photographed randomly for each dog with a photomicroscope (Olympus BX50, Olympus Optical, Tokyo, Japan) at 100x magnification to evaluate the percentage of medial thickness (%MT) by measuring the internal and external diameters. The percentage of the medial thickness was calculated by the following equation: (external diameter – internal diameter)/external diameter x 100 (17, 18). The 10 pulmonary arteries were photographed at 400x magnification at five per each pulmonary artery. The number of pulmonary arterial SMCs per 400x field were then counted and averaged. The number of cells positive for Bax, Bcl2 and caspase-3 and caspase-8 were counted and calculated as percentage of positive cells per total cell count, and the Bax/Bcl2 ratio was calculated by dividing the percentage of Bax-positive cells by the percentage of Bcl2-positive cells.

Statistical analysis

Statistical analysis was performed by a computer-based software (SPSS, IBM, Chicago, IL, USA). The normality test was evaluated by using the Shapiro-Wilk test. Data were expressed as mean ± standard deviation (SD). The differences among the control, DMVD and DMVD+PH groups were tested with one-way ANOVA. Bonferroni test was used for post hoc analysis. The p-value for pairwise comparison was adjusted by Bonferroni correction. An independent T-test was used for analyzing the difference of Bax/Bcl2 ratio between the DMVD and DMVD+PH groups. The correlations between %MT, the average number of pulmonary arterial SMCs and proteins associated with apoptosis pathways were evaluated by Pearson's correlation. P-value <0.05 is considered to be statistically significant.

List of abbreviations

DMVD	Degenerative mitral valve disease
LA/Ao	Left atrium to aorta ratio
LVIDdn	Left ventricular internal diameter during diastole
%MT	Percentage of medial thickness
PAP	Pulmonary arterial pressure
PH	Pulmonary hypertension
RHC	Right heart catheterization
SD	Standard deviation
SMCs	Smooth muscle cells

Declarations

Ethics approval and consent to participate

This study collected tissue samples from the carcasses of client owned dogs that their owner donated for necropsy and pathological study at Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University. An Informed consent form has been signed by the owners.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SDS designed the study. SS performed sample collection and immunohistochemistry. AR performed the histopathological examination. AR, SDS and SS analysed and interpreted data. All authors read and approved the final manuscript.

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Figures

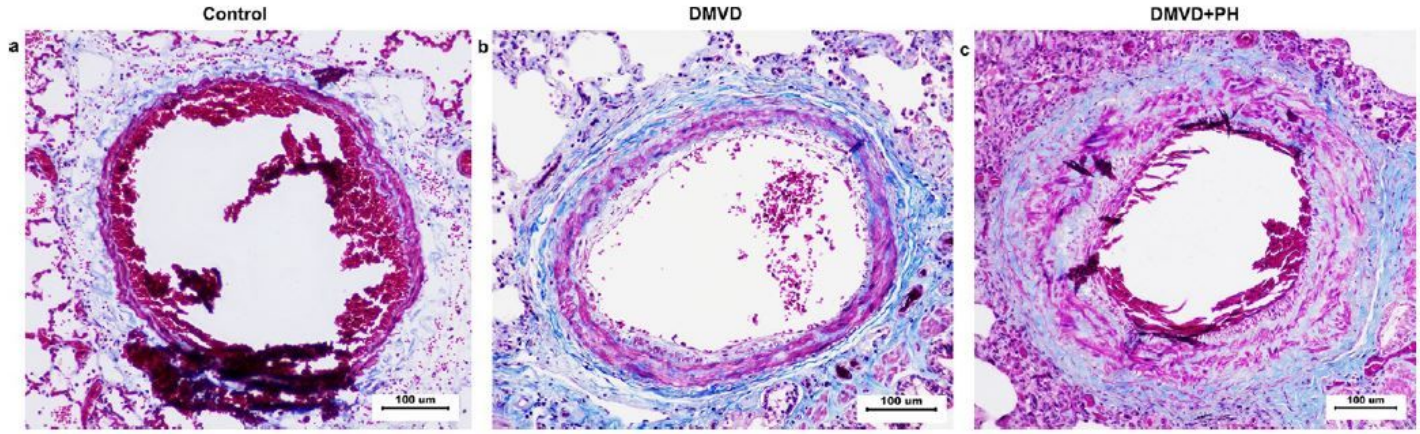

Figure 1

The histopathology of the pulmonary artery Masson's trichrome staining revealed normal thin medial layer in the control group (a) and increased medial thickness in the degenerative mitral valve disease (DMVD) (b) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) (c) groups. Red color are staining of SMCs; Blue color are staining of collagen fibers (Masson's trichrome stain, 100x magnification).

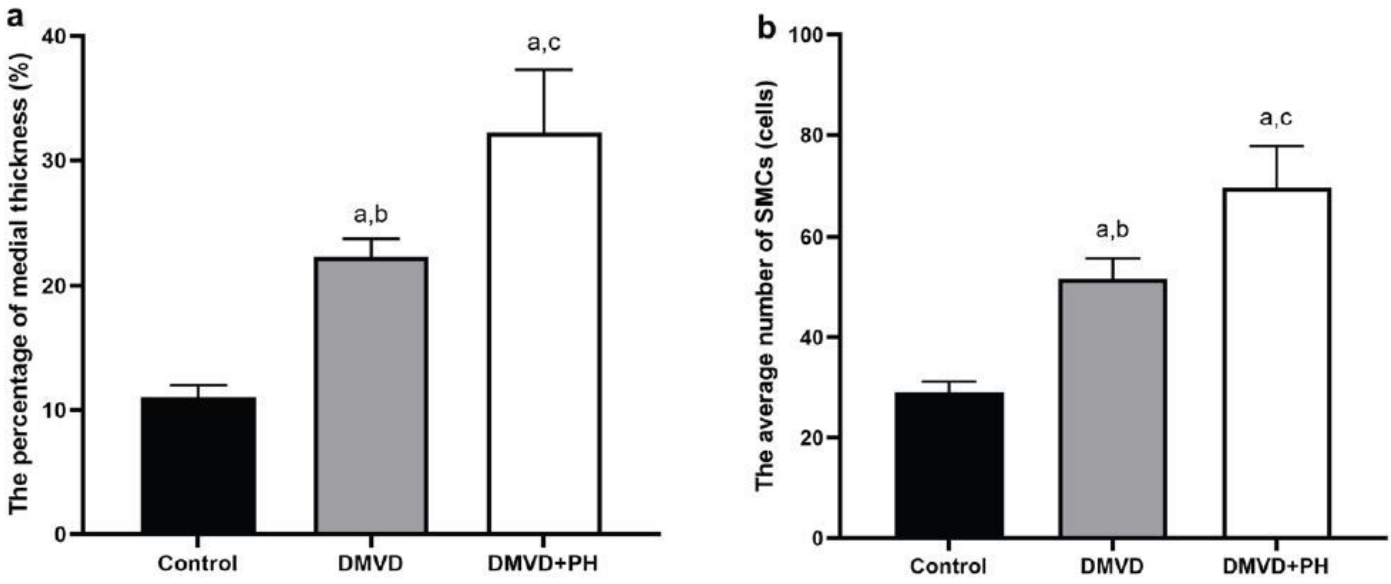

Figure 2

The percentage of medial thickness and the average number of pulmonary arterial SMCs per vessel. The graphs show the percentage of medial thickness (%MT) (a) and the average number of pulmonary arterial SMCs per vessel (b) of the control, degenerative mitral valve disease (DMVD) and degenerative

mitral valve disease with pulmonary hypertension (DMVD+PH) groups. Data are expressed as mean and standard deviation (SD) (bars). An increase in %MT in the DMVD and DMVD+PH groups conforms to an increase in the average number of pulmonary arterial SMCs. a Indicate statistically significant difference at $p < 0.05$ compared to the control group. b,c Indicate statistically significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.

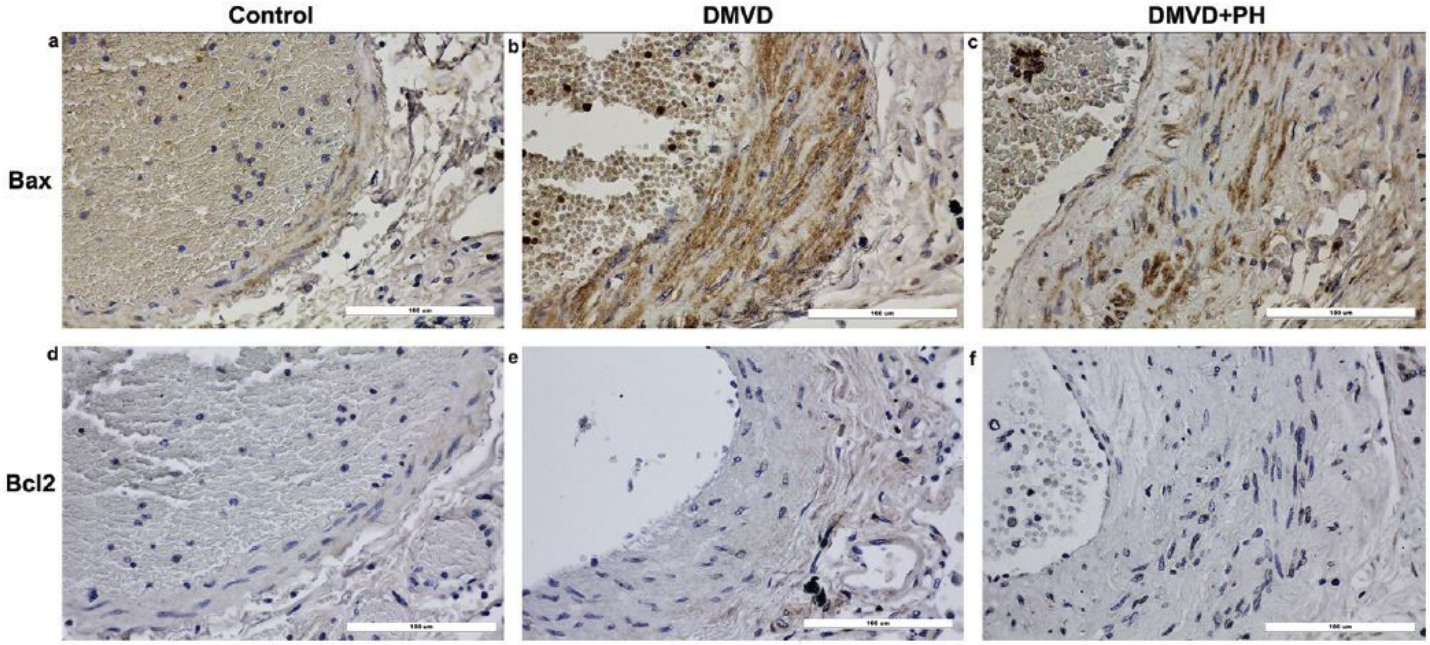

Figure 3

Immunohistochemical expression of Bax and Bcl2 in pulmonary arteries. The images shows the expression of Bax and Bcl2 in the pulmonary arteries of the control (a, d), degenerative mitral valve disease (DMVD) (b, e) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups (c, f). These proteins were mainly expressed in SMCs located at medial layer of pulmonary artery. Bax protein was stained in all groups, whereas Bcl2 protein was not stained in the control group. The intracytoplasmic staining of Bax and intranuclear staining of Bcl2 in pulmonary arterial SMCs presented as brown color (Labeled streptavidin-biotin, Immunohistochemistry, Mayer's Hematoxylin counterstained, 400xmagnification).

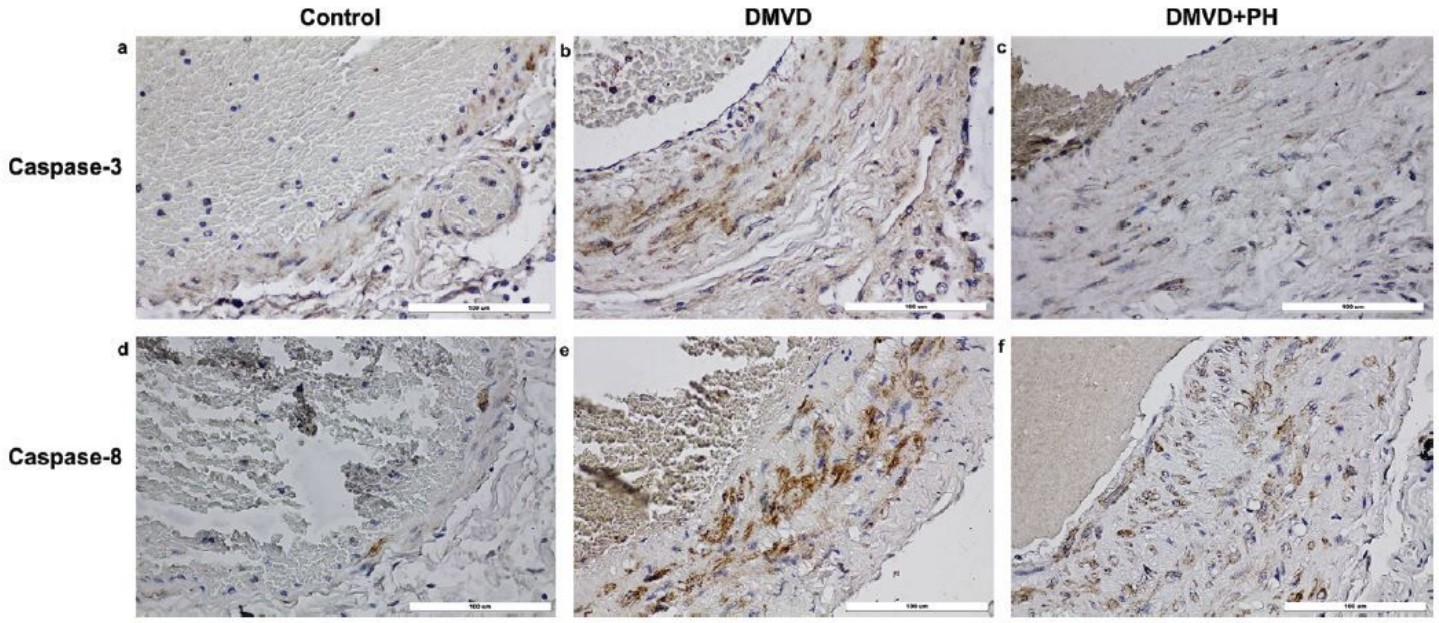

Figure 4

Immunohistochemical expression of caspase-3 and -8 in pulmonary arteries. The images show the expression of caspase-3 and -8 in the pulmonary arteries of the control (a, d), degenerative mitral valve disease (DMVD) (b, e) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups (c, f). The caspase-3 and -8 expressions were mainly found in SMCs located at the medial layer of the pulmonary artery in all 3 groups. The intracytoplasmic and intranuclear staining of caspase-3 and intracytoplasmic staining of caspase-8 were in brown color. (Labeled streptavidin-biotin, Immunohistochemistry, Mayer's Hematoxylin counterstained, 400x magnification).

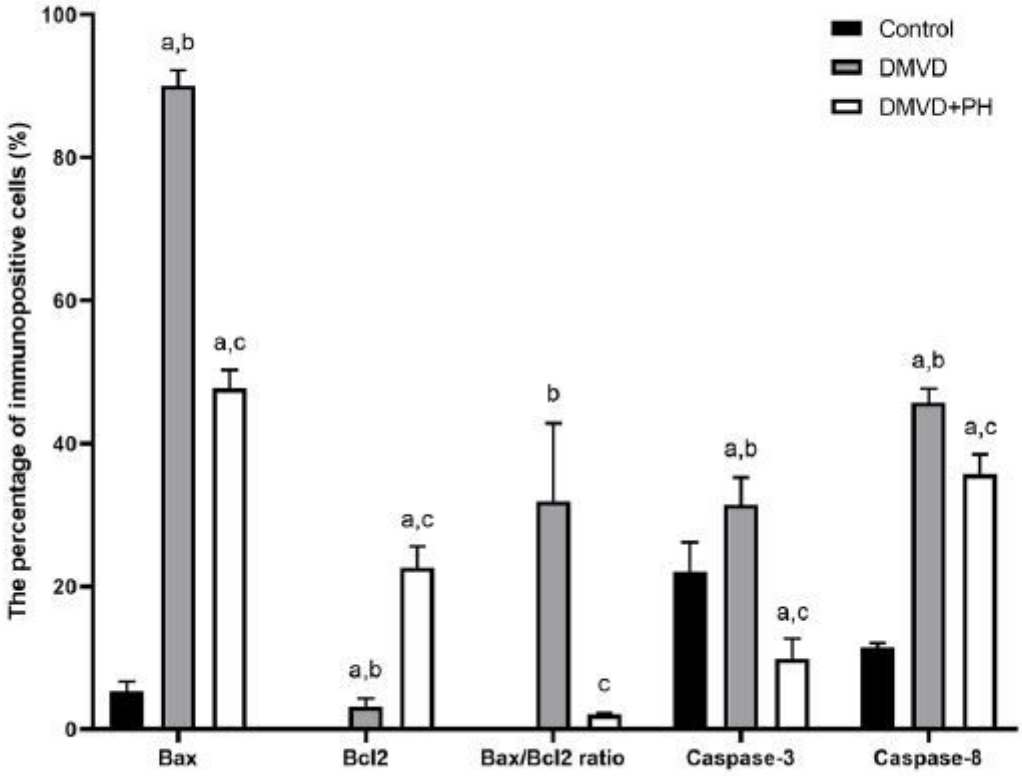

Figure 5

The percentage of Bax, Bcl2, and caspase-3 and -8 positive cells and Bax/Bcl2 ratio The graph show the percentage of Bax, Bcl2, and caspase-3 and -8 positive cells and Bax/Bcl2 ratio of the control, degenerative mitral valve disease (DMVD) and degenerative mitral valve disease with pulmonary hypertension (DMVD+PH) groups. Data are expressed as mean and standard deviation (SD) (bars). The percentage of Bax and caspase-3 and -8 positive cells as well as Bax/Bcl2 ratio were highest in the DMVD group, opposite to the percentage of Bcl2 positive cells that was highest in the DMVD+PH group. a Indicate statistically significant difference at $p < 0.05$ compared to the control group. b,c Indicate statistically significant difference at $p < 0.05$ between the DMVD and DMVD+PH groups.