Ameliorative effects of aminoguanidine on rat aorta in Streptozotocin-induced diabetes and evaluation of α-SMA expression

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ABSTRACT

Objective: Diabetes mellitus is one of the chronic metabolic diseases which is characterized by microvascular and macrovascular complications. This study was designed to investigate the improving the effects of amnioguanidine on aortic damage in a streptozotocin (STZ) induced diabetic rat model.

Methods: Thirty-two male Sprague-Dawley rats divided into four groups as follows: Control, Aminoguanidine, Diabetes, and Diabetes+Aminoguanidine. Experimental diabetes was induced by single dose STZ (45 mg/kg) intraperitoneally. After administration of STZ, the DM+AMG group began to receive AMG (1 g/L) was prepared by dissolving in tap water during 10 weeks. At the end of the study, blood glucose levels were determined and rats were sacrified by ketamine anesthesia. Following routine tissue process, aortas were embedded in paraffin. Histochemical (H-E and Orcein) and immunohistochemical α-smooth muscle actin (α-SMA) stains were applied and the sections examined by light microscope. Statistical analysis was carried out using the SPSS 13.0 statistical program.

Results: The rats in diabetes group had significantly higher blood glucose levels than the rats of control. The main histological alterations were detected in tunica media such as extensive thickening (414.32±9.62 μm), irregular of elastic fibers and intensive α-SMA staining in diabetic rats. The thickness of tunica media was statistically increased in DM group, when compared with the control group ($p<0.001$). On the other hand, AMG prevented disorganization of elastic fibers and overexpression of α-SMA. The mean thickness of tunica media was decreased significantly in DM+AMG (319.16±6.53 μm) compared with the DM group ($p<0.001$).

Conclusion: Our results demonstrate that AMG treatment may protect the impairment of aort structure at histological level.

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Key words: diabetes, aminoguanidine, α-smooth muscle actin, nitric oxide, aorta, rat

Introduction

Diabetes mellitus is one of the chronic metabolic diseases, which manifested by an imbalance in maintenance of blood glocose level and increased oxidative stress (1, 2). Diabetes is characterized by the occurrence of multiple serious vascular complications such as atherosclerosis and hypertension (3-5). Atherosclerotic process, pathologically manifested as arterial narrowing due to medial calcification and thickening, is the major feature of diabetic macroangiopathy (6). Proliferation of vascular smooth muscle cells (SMC) plays a central role in the development of atherosclerosis (7). Alpha-smooth muscle actin (α-SMA), an isoform of SMC and present in high amounts in vascular SMC. It has been demonstrated in the cytoplasm of pericytes in rat and human capillaries and venules by immunocytochemical methods (8).

Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, is the most widely used agent in experimental diabetes (9, 10). This agent has been used for induction of diabetes in animals as a model of insulin-dependent diabetes mellitus (Type 1 diabetes mellitus) (9). Many actions have been attributed to STZ that are including damage to the pancreatic β-cell membrane and decreases insulin secretion from the β-cell. In addition, STZ has been shown to induce DNA strand breaks and methylation in pancreatic islet cells (9, 11, 12).

The alteration in nitric oxide (NO) has been associated with the pathogenesis of chronic vascular complications in diabetes (13). Aminoguanidine (AMG), a phenylhydrazine compound, reduces

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NO production by selectively inhibiting the synthesis of nitric oxide (14, 15). Experimental studies shown that AMG inhibits inducible nitric oxide synthase (iNOS) and reduce nitrite over formation, attenuating the pathological alteration in the rat glomerular capillary (16, 17). Therefore AMG may have preventive effective on the development of diabetic cardiovascular complications.

To our knowledge, a study related to effects of AMG on impaired vascular structure of diabetic rats in literature was not found. This study was designed to determine whether AMG, inhibitor of NO, can cause curative structural alterations in the aorta of diabetic rats.

Methods

Animals and experimental design

The study was performed on 32 male Sprague-Dawley rats provided by the İnönü University Animal Research Center. The animals were housed in individual cages for 10 weeks in a well ventilated room with a 12: 12-hour light/dark cycle at 21°C. Animals were fed with standard rat chow and tap water *ad libitum*. All experiment were approved by the Ethics Committee for Animal Experiments of İnönü University Faculty of Medicine and followed the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals (Ethic no: 2009/47).

The animals were randomly divided into four groups. There were 8 rats in each groups, as follows: Control (C), AMG, Diabetes (DM), and DM+AMG. STZ (Sigma, St. Louis, MO), freshly dissolved in 0.9% saline, was injected intraperitoneally at a single dose of 45 mg/kg. Three days after STZ injection, blood glucose levels were measured using reagent strips (Accu-Check Active Glucose test strips, Roche, Germany) with a glucometer (Accu-Check Active, Roche, Germany) in samples obtained from the tail vein. Animals with blood glucose levels of 270 mg/dL and above were accepted as diabetic rat. AMG, 1 g/L, was prepared by dissolving in tap water and received during 10 weeks.

Histopathological evaluations

The rats of all groups were sacrified by ketamine anesthesia at the end of the study. The descending aorta were quickly removed. The tissue samples were fixed in 10% formalin for 48 h and prepared for routine paraffin embedding. Sections were cut at 5 µm, mounted on slides, stained with hematoxylin-eosin (H&E) and Orcein. The thickness of tunica media was measured under 40X objective magnification and five points of aorta were selected randomly. For immunohistochemical analysis, α-SMA antibody (Labvision, USA) was used according to the manufacturer's instructions. Density of staining was graded from 0 to 3: 0 (no staining), 1 (weak), 2 (moderate), 3 (strong). For this analysis, each slide was observed under 40X objective magnification. All sections were evaluated using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical analysis

Statistical analysis was carried out using the SPSS 13.0 statistical program (SPSS Inc., Chicago, Ill., USA). Normality for continued variables in groups were determined by the Shapiro-Wilk test. The Kruskal-Wallis and Mann-Whitney U tests were used for comparison of blood glucose levels and intensity of α-SMA among the studied groups. Results are expressed as median (min-max). The thickness of tunica media were analyzed by one-way ANOVA. Post-hoc comparisons were performed using LSD test. Results are expressed as mean±SE. A $p<0.05$ was regarded as significant.

Results

Histopathological findings

Control group: The aorta of the control sections were in normal histological appearence. The tunica intima of the aorta was composed of a single layer of endothelial cells. The tunica media also showed a regular appearance, including smooth muscle cells, between the distinct elastic lamina which were wavy and arranged concentrically (Fig. 1a). Tunica media thickness was measured as 298.22±2.62 µm in this group. Elastic lamellae appeared purple/black in Orcein stained slides (Fig. 1b). With anti-α-SMA labeling, smooth muscle cells were seen in the interspaces between the concentric lamellae (Fig. 1c).

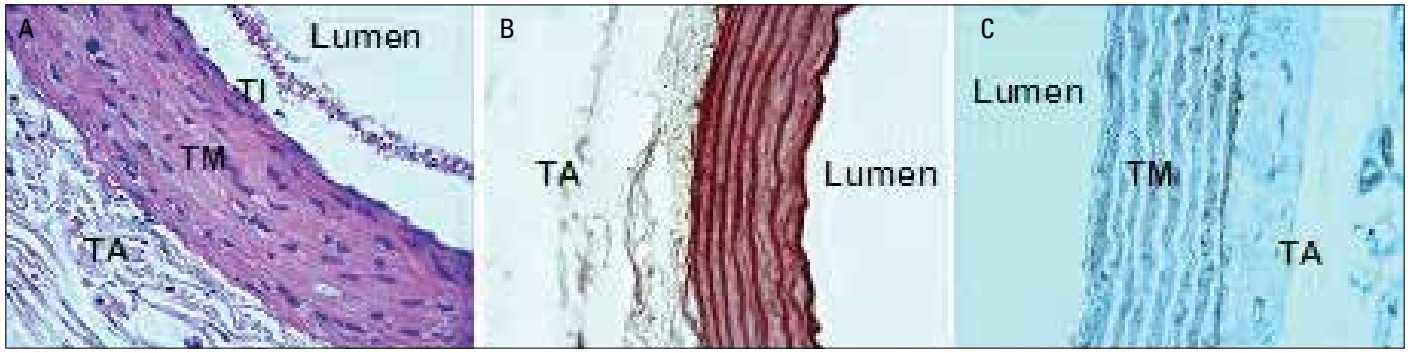

Figure 1. A-C. Photomicrographs of control group. (A) Histological appearence of aorta was normal. Tunica intima (TI), tunica media (TM) and tunica adventitia (TA) were observed, H-E; X40. (B) Histological appearence of aorta was normal. Elastic laminae were observed, Orcein; X40. (C) Histological appearence of aorta was normal, α-SMA; X40.

AMG group*:* The histological appearance of AMG group was similar to control group except tunica media thickening (Fig. 2a, b). The mean thickness of tunica media was 343.93±3.57 μm in AMG group. In anti-α-SMA stained sections, smooth muscle cells were seen among concentric lamellae (Fig. 2c).

DM group: STZ-induced diabetes caused severe alterations in the structure of the vascular wall. The tunica intima of the aorta was irregular. Extensive thickening (414.32±9.62 μm) and disorganization were observed in tunica media. When compared with the control group, the thickness of tunica media was statistically increased in DM group ($p<0.001$). Tunica media showed irregular smooth muscle cells. In addition, smooth muscle cells with perinuclear halo were seen in the tunica media (Fig. 3a). The elastic lamellae were flattened in Orcein stained sections (Fig. 3b). The tunica media showed strong intensity of anti-α-SMA labeling when compared with the control group ($p<0.05$) (Fig. 3c).

DM+AMG group*:* The mean thickness of tunica media was significantly decreased in DM+AMG (319.16±6.53 μm) when compared with the DM group ($p<0.001$). However, AMG administration did not completely ameliorate thickness of tunica media. There was a significant difference between control and DM+AMG group ($p<0.05$). AMG treatment of diabetic rats reduced smooth muscle cell degeneration. The elastic lamellae of tunica media were preserved in this group (Fig. 4a, b). Although, immunoactivity of anti-α-SMA labeling decreased with respect to DM group, there was no statistically significant difference between these groups ($p>0.05$) (Fig. 4c).

The tunica adventitia showed normal histology in all experimental groups. The tunica adventitia consists of collagen and elastic fibres, fibroblasts, vasa vasorum and nerves. The mean thickness of tunica media of all groups was shown in Table 1. Positive immunostaining for α-SMA of all groups was shown in Table 2.

Blood glucose levels

A significant increase in blood glucose levels was observed in DM group (587.0±5.0 mg/dL) at the end of the 10th week. On the other hand, the DM+AMG rats (334±7.8 mg/dL) had significantly lower blood glucose levels than DM group ($p<0.01$) (Fig. 5).

Discussion

In the present study, we aimed to demonstrate the effects of STZ-induced diabetes on aorta as well as the possible ameliorative effect of AMG. We have evaluated blood glucose levels and aorta histopathology in diabetic rats. Histological changes were examined via light microscopy. Our first notable finding was the ameliorative effect of AMG on blood glucose level. Pathological documents shows that patients with diabetes are at high risk for several cardiovascular disorders (18). These changes have been

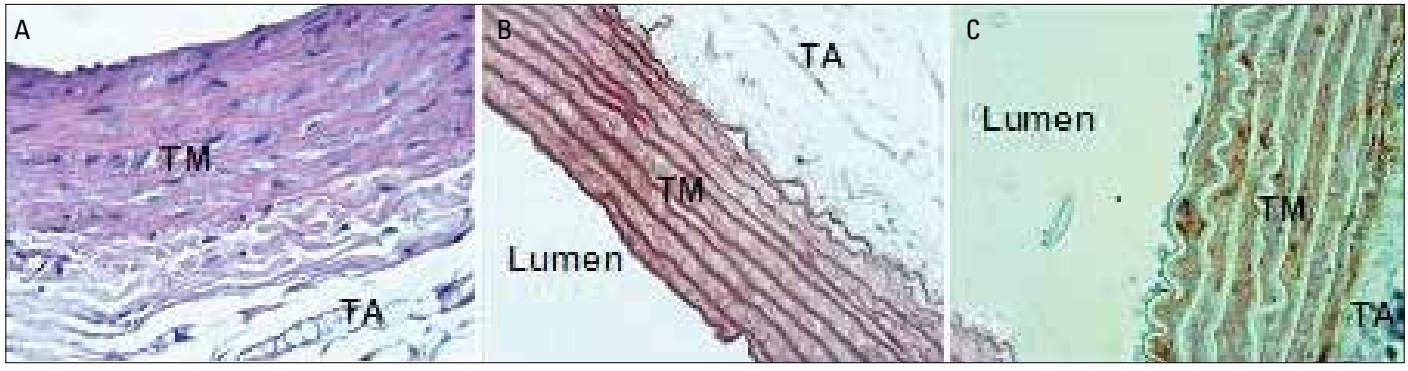

Figure 2. A-C. Photomicrographs of AMG group. Tunica media (TM) and tunica adventitia (TA) are observed normal. Histological appearence was similar to control group. (A) H-E; X40. (B) Orcein; X40. (C) α-SMA; X40

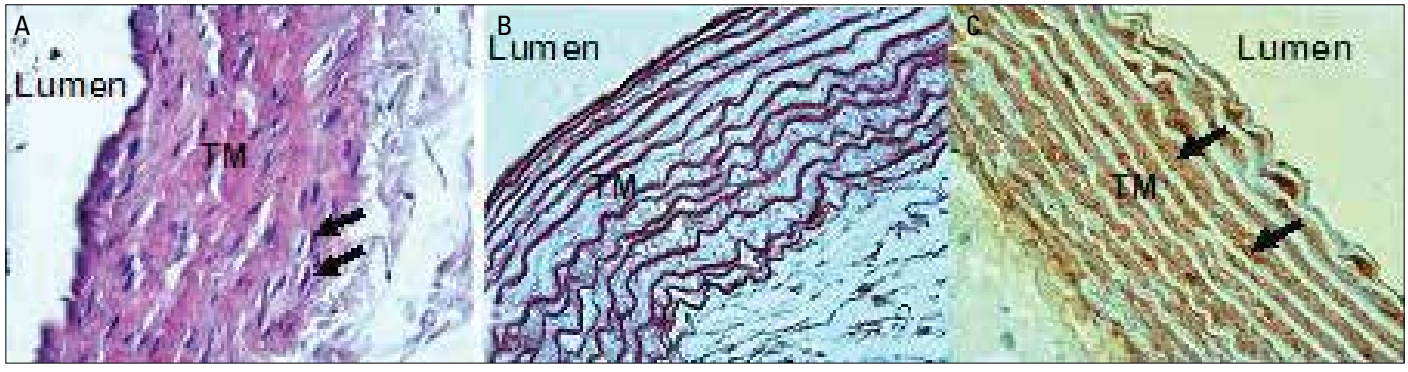

Figure 3. A-C. Photomicrographs of DM group. (A) Tunica media showed irregular smooth muscle cells. Smooth muscle cells with perinuclear halo (arrows) were seen in the tunica media (TM), H-E; X40. (B) Disorganization in tunica media (TM) were observed, Orcein; X40. (C) The tunica media showed strong intensity of anti-α-SMA labeling (arrows), α-SMA; X40.

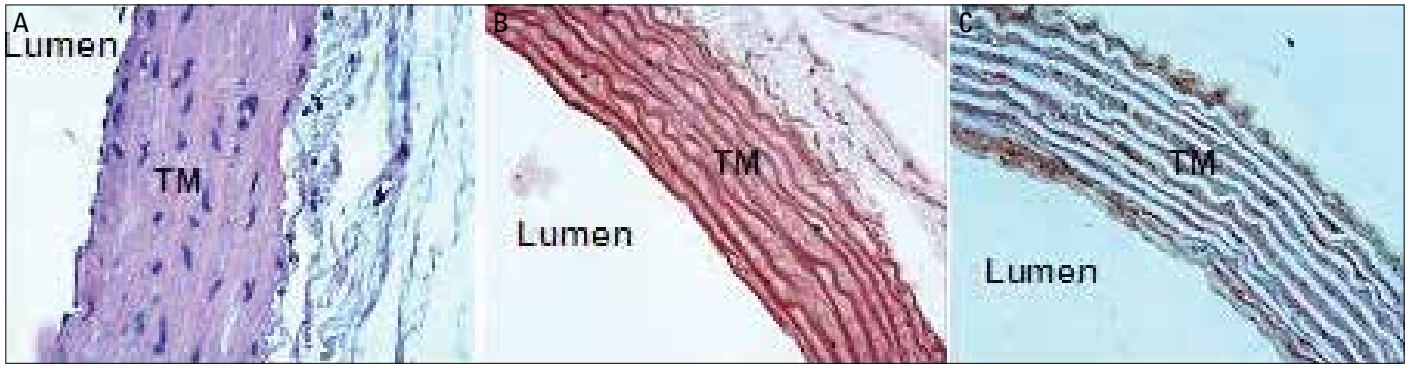

Figure 4. A-C. Photomicrographs of DM+AMG group. (A) Histological alterations were reduced. The appearence of tunica media (TM) is almost normal, H-E; X40. (B) Elastic lamellea in tunica media (TM) is almost normal. Orcein; X40. (C) Immunoactivity were reduced, α-SMA; X40

Table 1. The mean thickness of tunica media for all groups

	Thickness of tunica media, μm
Control	298.22±2.62
AMG	343.93±3.57[a]
DM	414.32±9.62[b]
DM + AMG	319.16±6.53[c,d,e]

Data are expressed as arithmetic mean±SE.
[a]p<0.001 vs. control group, [b]p<0.001 vs. control and AMG group, [c]p<0.05 vs. control group, [d]p<0.01 vs. AMG group, [e]p<0.001 vs. DM group

Table 2. Positive immunostaining for α-SMA of all groups

	Slight	Moderate	Strong
Control	0 (0-0)	2 (2-2)	0 (0-0)
AMG	0 (0-0)	3 (3-3)[a]	0 (0-0)
DM	0 (1-0)	4 (5-0)	1 (5-0)[c]
DM + AMG	0 (3-0)	3 (5-2)[b]	0 (3-0)

Data are expressed as median (min-max).
[a]p<0.001 vs. Control group, [b]p<0.01 vs. Control group, [c]p<0.05 vs. Control and AMG group

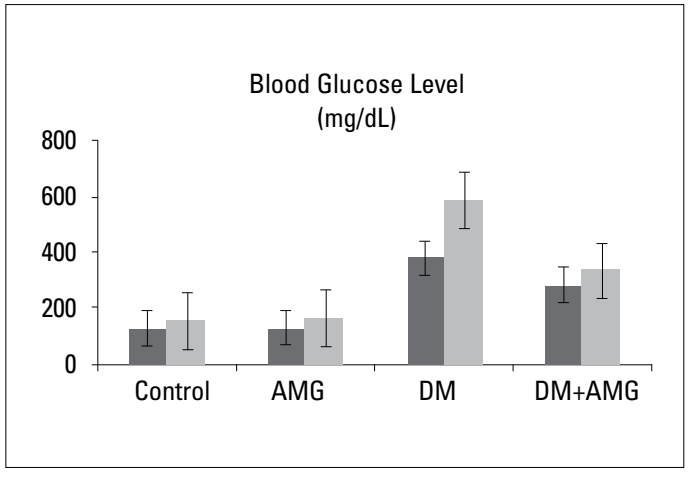

Figure 5. The mean blood glucose levels of all groups. In DM group, significantly elevated when compared to the control group (p<0.01). In DM+AMG group, significantly decreased when compared to DM group (p<0.01)

linked to vascular metabolic derangements associated with hyperglycemia (19). STZ, when injected into an experimental animal, induce a blood glucose response, which is accompanied by corresponding inverse changes in the plasma insulin concentration (20). Ten weeks after STZ injection in our study, blood glucose levels in DM group were markedly higher than control groups. On the other hand, AMG significantly lowered blood glucose levels. These findings are in agreement with other researchers (21-24), they reported that AMG was decreased blood glucose levels in diabetic rats.

Another major finding of our study was the aortic damage in DM group. The effects of diabetes on vascular complications have been studied in different blood vessels (19). The morphological findings of this study showed that the structural organization of the aorta was distrubuted in STZ-induced diabetic rats. In addition, proliferation of smooth muscle cells (SMCs) in the tunica media was observed in diabetic group. STZ injection significantly increased thickness of tunica media in this study. In human studies tunica media thickness of aorta increased significantly in diabetes compared with the healthy people (25-27).

Diabetes may contribute to extracellular matrix (ECM) production and smooth muscle proliferation by a variety of mechanisms. High levels of glucose can impair endothelial cell replication and accelerate cell death of endothelial cells in culture (28). α-SMA is the most wide cellular protein of SMCs and it has been commonly used to identify the SMC development in response to injury and in disease (29). In our study, we examined from the view point of histologic change of aortic SMCs. Using immunohistochemistry, we demonstrated that increase intensity of α-SMA labeling in diabetic rats when compared to the control group. In diabetes, protein kinase C (PKC) activates via the glycolysis system. PKC activation is related to proliferation of SMCs as well as accelerated synthesis of ECM proteins, and thus plays significant roles in the inception and progression of vascular cell dysfunction in diabetes mellitus (30). A variety of molecules have been shown to stimulate proliferation of SMCs. Among the most potent growth factor for SMCs in culture is platelet derived growth factor (PDGF) (31, 32). PDGF is powerful SMC mitogen resulting in SMC migration and proliferation (28, 33) and released from

injured endothelial cells in diabetics (28). In previous studies, researchers have reported that arterial SMCs and medial layers of arteries of diabetic rats express more PDGF β-receptor than those of control rats (32, 34, 35). Poulter et al. (36) and Askari et al. (31) suggested that proliferation of SMCs in the tunica media caused a increase in collagen synthesis.

The other findings in this study were irregularity and fragmentation of elastic fibers. El-Kassaby et al. (37) reported that decreased elastin synthesis by SMCs caused disruption of elastic fibers in diabetes.

Administration of AMG ameliorated thickness of aortic wall and organization of elastic fibrils. Furthermore when AMG administered concomitantly with STZ, α-SMA expression reversed the attenuated responses. Taken together, it might be speculated that AMG does not effect α-SMA expression in healthy rat aortas, but reverses diabetes-induced α-SMA activity, possibly through a decrease in free radical formation. The decrease in free radical formation prevents smooth muscle injury induced by diabetes and renews thickness of tunica media.

In previous studies, benefit of antioxidants on vascular function demonstrated in experimental diabetes. Ting et al. (38) showed that high doses of vitamin C can improve vascular disfunction in diabetes. AMG has a strong antioxidant and free radical scravenging (24). AMG supplementation affects vascular morphology involves its antioxidant properties. On the other hand, AMG inhibits iNOS selectively and reduces NO production (2, 39, 40). NO is an important mediate for controlling vascular resistance and is responsible for vasodilatation (41). Furthermore, NO plays a major role in many pathophysiological changes occurring in diseases such as diabetes, hypertension, and atherosclerosis (13, 17). It is known that after STZ administration to rats and mice, there is an increase in nitrite and nitrate, the end products of NO metabolism (17). We think that inhibition of NO production play a role in the reduction of thickness of tunica media and arrangement of elastic lamellae following AMG treatment.

Study limitations

This study was designed to investigate whether aminoguanidine can ameliorate diabetic aorta damage in rats. The major limitation of the present study was that biochemical enzyme levels in tissue and serum couldn't being measured. Another limitation was NO levels in tissue and serum couldn't be measured biochemically. Further study will be done to measure the biochemical parameters in tissue and serum. Also, the possible mechanism of AMG ameliorate diabetic cardiac injury will be clarified in the further study.

Conclusion

To our knowledge, however, a study about available of effects AMG on impaired vascular structure of diabetic rats in literature was not found. Therefore, in this study, we evaluated α-SMA expression and the potential effects of AMG treatment on the thickness of tunica media in diabetic aorta. The present study showed that statistically a significant increase in thickness of media in diabetic groups. On the other hand, AMG had beneficial effects against histological injury induced by STZ treatment. We found that AMG reduce the medial thickening. However, further research is needed on long term uses of AMG in order to show its beneficial effects on diabetic complications.

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Authorship contributions: Concept - N.V.; Design - N.V., D.O.; Supervision - H.E., N.V.; Resource - H.E.; Materials - D.O.; Data collection &/or processing - D.O.; Analysis &/or interpretation - H.E., E.T.; Literature search - E.T.; Writing - H.E., N.V.; Critical review - N.V.; Other - A.Y.

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