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EXPERIMENTAL STUDY

Coronary angiogenesis during morphine and nicotine withdrawal in two-kidney one clip hypertensive (2K1C) rats

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ABSTRACT
OBJECTIVE: This study was aimed to investigate the effects of addiction to nicotine and morphine and their withdrawal on coronary angiogenesis and serum NO concentrations in two-kidney one-clip hypertensive (2K1C) rats.
METHODS: Male hypertensive rats were divided into the two below groups: Group (1): Rats received saline for 8 weeks (n = 8); Group (2): Rats received morphine and nicotine for 8 weeks (n = 32). At the end of 8 weeks, the groups (2) were divided into the four sub-groups, which three of them were treated with withdrawal drugs. Following treatments, blood pressure, heart rate, plasma renin activity (PRA), NO concentration and capillary density were measured.
RESULTS: Results showed that blood pressure was significantly reduced in the addicted group when compared to non-addicted ($p < 0.05$). The withdrawal completely reversed blood pressure to the level observed pre-addiction ($p < 0.05$). Coronary angiogenesis was significantly lower in the addicted group in comparistion to normal ($p < 0.05$) but withdrawal of addiction did not improve angiogenesis.
CONCLUSION: On the basis of the present findings, it may be indicative that the risk of cardiovascular complications in addiction is concurrent to chronic hypertension, which shows the importance of early diagnosis and treatment in clinical condition *(Fig. 4, Ref. 59)*. Text in PDF *www.elis.sk.*
KEY WORDS: hypertension, angiogenesis, morphine, nicotine, withdrawal.

Introduction

Neovascularization or angiogenesis is described as a phenomenon, in which new vessels are generated from pre-existing blood vessels. The event of angiogenesis occurs in response to angiogenic molecules such as chemokines and hypoxia that result from tissue injury (1).Angiogenesis is important in development of cardiovascular system (2).Vascular endothelial function is well correlated with angiogenesis and the endothelium stimulates the processes of vasodilation, inflammation and vascular smooth cell proliferation by releasing nitric oxide (NO) (3). NO as a biological mediator, is involved in an array of physiological and pathophysiological processes in various organs. Potentially, NO is a regulator for neovascularization, which is evidenced by many studies. Accumulating data also are demonstrative that NO is closely involved in the regulation of systemic blood pressure (4).

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Hypertension is a well-established risk factor for cardiovascular disorders. It is also indicated that hypertension is associated with several vascular abnormalities, including endothelial dysfunction, microvascular rarefaction and remodeling (5, 6). Hypertension is associated with defected blood vessel growth (6, 7), so that impaired angiogenesis is reported in hypertensive patients by some studies (8).While others showed increased angiogenic factors in serums of hypertensive individuals (9). In other words, impaired angiogenesis during embryonic development results in undeveloped vascular system that precedes hypertension in later life (6). Nicotine is the addictive compound present in tobacco plant and in turn smokes (10). Nicotine is an emerging leading cause for cardiovascular disorders in the most of developed countries (11–13). Vascular endothelial dysfunction (VED) has been considered as a hallmark of various cardiovascular disorders (14, 15). Long-lasting exposure to nicotine induces the risk of VED via decreasing the generation and bioavailability of nitric oxide (NO) (16, 17). Morbidity and mortality from cardiovascular disorders are also elevated when smoking is associated with hypertension (18). Opioid receptors, including MOR, are expressed on endothelial cells surfaces (19) and in the vascular endothelium, morphine activates NO via MOR, what in turn leads to vasodilatation(20), promote cell proliferation (20) as well as angiogenesis induced by the endothelial cell specific growth (21).

According to the afore mentioned documentary facts in the present investigation, we evaluated the effects of addiction to morphine and nicotine as well as chronic hypertension (2K1C) and

those withdrawal on blood pressure, coronary angiogenesis and serum NO concentration.

Material and methods

Animals

Forty male Wistar rats (aged 10–12 weeks and weightind 200–250 g) were obtained from the Pasteur Institute of Iran, Tehran. Animals were kept in animal house with 12 h light/dark cycle. The animal house temperature was maintained at 20–25 °C. All animals had free access to food and water during experiments. The ethical committee of the Rafsanjan University of Medical Sciences approved the project and animals were handled with care and by staff who had animal handling license.

Experimental protocol

Following one week of habituation to animal house, male hypertensive rats were randomly divided into two following groups: Group (1): Hypertensive rats that only received solvent of saline for 8 weeks (n = 8); Group (2): Hypertensive rats receiving 3 mg/kg of morphine and 0.01 mg/kg of nicotine for 8 weeks (n = 32). At following 8 weeks, the main groups were divided into four sub-groups, where hree of the sub-groups (n = 24) were on withdrawal: A sub group deprived from morphine (n = 8), A sub group deprived from nicotine (n = 8), A sub group deprived from both morphine and nicotine (n = 8). After 24 hours of withdrawal, rats were treated with naloxone (2 mg/kg). Direct blood pressure was measured, blood sample was taken and coronary angiogenesis was evaluated.

Induction of hypertension in rats by 2K1C goldblatt method

Initially rats were anesthetized using intraperitoneal ketamine-hydrochloride (60 mg/kg) and xylazine(7.5 mg/kg). The left kidney was exposed via flank incision and a silver clip within internal gap of 0.2 mm was located around the renal artery. To control the risk of infection, rats received prophylactic treatment by penicillin G (25000 IU/rat) after surgery. Systolic and diastolic blood pressure was measured once a week by a tail-cuff plethysmography under light ether anesthesia. Subsequent to 8 weeks of treatment, animals were anesthetized and direct blood pressure was measured by a catheter (PE50), inserted into femoral artery.

Plasma renin activity

The plasma renin activity (PRA) was measured using Gamma coat PRA Radioimmunoassay kit (DiaSorin Inc). PRA was measured after withdrawal in hypertensive rats.

Measurement of NO concentration

The serum concentrations of NO were measured by Griess reagent system (Promega Corporation, Madison, USA), using available reagents as previously described (23). In brief, serums were added into wells (96-well enzymatic assay plate). Sulfanilamide solution was added to all collected samples and then *N*-1-naphtylethylenediamine dihydrochloride under acidic conditions was added. The absorbance was detected by a microplate reader in 520–550 nm wavelengths. A standard curve was generated and the concentration of NO in serum samples was determined by comparison to NO standard level. The sensitivity of detection was 2.5 μM.

Evaluation of capillary density

The tissue samples were obtained from the hearts following weeks of the induction of hypertension. Frozen tissue sections with 5 μm thickness were prepared from each sample. Endothelial cells were stained by immunohistochemical staining methods using, mose anti-rat CD31 monoclonal antibody (Abcam, Abingdon, UK). Capillary density was evaluated by counting twenty random microscopic fields (magnification × 400) from three different sections in each tissue block by three blind examiners. Capillary density was expressed as the number of CD31+ cells per square millimeter (mm2).

Statistical analysis

One-way ANOVA was used for comparison of data between groups. Data are expressed as the mean ± S.E.M. P value less than 0.05 was considered statistically significant.

Result

Blood pressure

The results of the present study showed lower blood pressure in hypertensive rats, which were addicted to morphine and nicotine in comparision to their status before addiction ($P<0.05$). After withdrawal, the blood pressure in morphine and nicotine withdrawal rats was significantly increased and went back to its level of before addiction ($p < 0.05$) (**Fig. 1**).

PRA measurement

The PRA levels were measured in all experimental groups in nicotine withdrawal animals. The PRA levels were decreased compared to control ($p < 0.05$) **(Fig. 2)**.

Serum NO concentration

Our results showed that there was no significant difference in serum NO concentration in pre-addict and addict to concur-

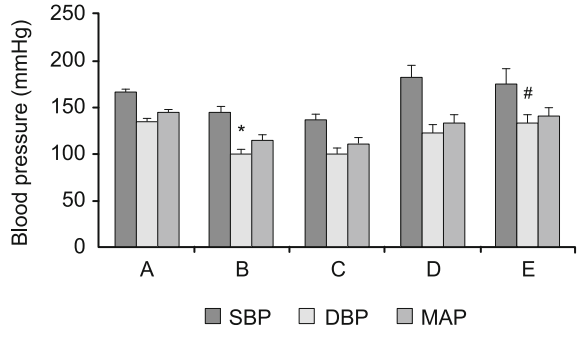

Fig. 1. Demonstrates variations in blood pressure in all experimental groups during addiction and withdrawal. * p < 0.05 vs pre-addict. # p < 0.05 vs nicotine withdrawal. A) pre-addict, B) addict, C) withdrawal-morphine, D) withdrawal-nicotine, E) addict-withdrawal

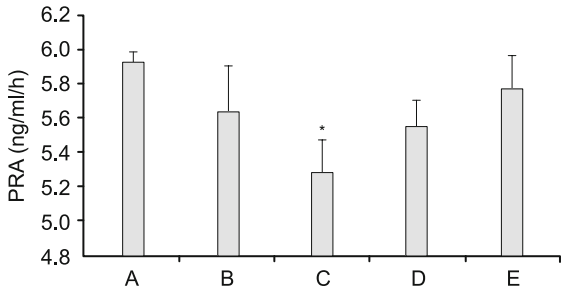

Fig. 2. Demonstrates PRA values (ng/ml/h) in experimental groups. * p < 0.05 vs pre-addict. A) pre-addict, B) addict, C) withdrawal-morphine, D) withdrawal-nicotine, E) addict-withdrawal.

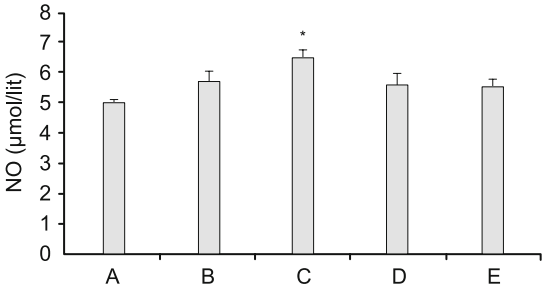

Fig. 3. Demonstrates serum level of NO in experimental groups during addiction and withdrawal. * p < 0.05 vs pre-addict. A) pre-addict, B) addict, C) withdrawal-morphine, D) withdrawal-nicotine, E) addict-withdrawal.

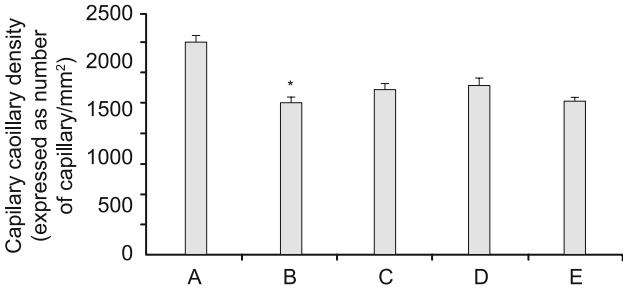

Fig. 4. Demonstrates capillary density (expressed as number of capillary/mm^2) of heart during addiction and withdrawal. * p < 0.05 vs pre-addict. A) pre-addict, B) addict, C) withdrawal-morphine, D) withdrawal-nicotine, E) addict-withdrawal.

rent morphine and nicotine group, while after withdrawal, serum NO concentration in nicotine withdrawal animals was increased and it was significantly higher than pre-addict group ($p < 0.05$). The withdrawal did not improve serum NO level and it was not altered compared to addict to concurrent morphine and nicotine animals (**Fig. 3**).

Capillary density

As illustrates in figure 5, capillary density (expressed as the number of capillary CD31$^+$cell per mm^2) was significantly lower in morphine and nicotine addicts when compared to pre-addict in hypertensive animals ($p < 0.05$).The addiction withdrawal did not alter capillary density in addicted hypertensive rats ($p > 0.05$).

Representatives of histological sections are shown in **(Figs 4 and 5).**

Discussion

Previous evidences confirmed that in the early phase of 2K1C hypertensive model, the elevated activity of renin-angiotensin-aldosterone system is the main responsible mechanisms involved in rising blood pressure (24, 25). Although, after 8 weeks of clipping, altered vascular structure is important in maintenance of hypertension (24). But chronic morphine treatment showed to decline baseline cardiovascular parameters MAP and HR. During morphine withdrawal, when naloxone was injected in morphine-treated rats, MAP and HR (26), systolic and diastolic BP were all attenuated (27). Evidences are in fader of the fact that smoking causes induced HR, SBP, DBP, and MBP (28).The increased BP following administration of nicotine may possibly be due to the induced PRA level (29). And other study demonstrated that at baseline, participants had lower resting HR, SBP, and DBP during the nicotine withdrawal (30). The results of the current study demonstrated that following the withdrawal, BP completely went back to the pre-addiction level. The microvascular density during development of hypertension have been documented in several experimental studies (31–33). Another study performed in hypertensive rats showed lower both capillary and arteriolar density in the heart of young animals (33). Present results are indicative of the fact that coronary angiogenesis decreases in hypertensive rats addicted to both morphine and nicotine. Recent evidence pointed to the involvement of morphine in angiogenesis, because endothelial cells expressed opioid receptor(s) (34) and responded to opiates by increasing intracellular calcium concen-

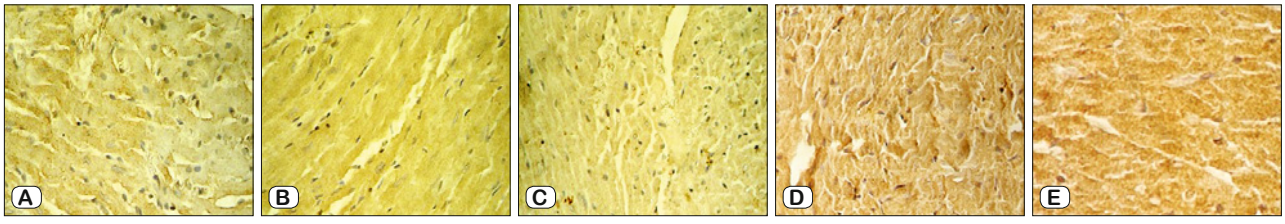

Fig. 5. Depicts a representative photograph of the cross section of the hearts in experimental groups. Original magnification: × 400. A) pre- addict (control), B) Administration of morphine + nicotine (addict), C) withdrawal-nicotine, D) withdrawal-morphine, E) morphine + nicotine withdrawal (addict. withdrawal).

tration and NO production (35). Accordingly, evidence showed that morphine enhanced endothelial cell proliferation and tube formation in vitro and increased in vivo blood vessel formation in a Matrigel plug assay (34). These studies revealed that morphine up regulated angiogenesis in sham and 2K-1C animals via generation of NO and angiogenesis in hypertensive animals occurred much more than what happened in sham group (36). Morphine acts both directly and indirectly on angiogenesis (37), however, the results are controversial. In contrast to all literature reports on the proangiogenic effects of morphine, high concentration of morphine is toxic for endothelial cells in vitro (35) and lead to oxidative stress-mediated endothelium dysfunction in vivo (38). Furthermore, morphine reduced wound healing and mobilization of endothelial progenitor cells in mice and decreased the formation of capillaries in vitro and in vivo matrigel plug assay (39). In animals, short-term exposure to nicotine augmented tumor vascularity, plaque neovascularization and retinal angiogenesis (40). Nicotine enhanced another type of vasodilating molecule associated with proliferation, migration and survival of EC (41–43), in fact increased angiogenesis. Some other workers suggested that nicotine could be toxic to endothelial cells (44), however, these observations were made using nicotine overdose, which were above clinically relevant concentrations (45). The acute effect of nicotine on capillary density was completely abrogated by chronic exposure to nicotine. That claimed to be mediated in part by down regulation of the vascular α7-nAChR, (an inhibition of NO synthase expression), as well as reduced in plasma VEGF levels (46). Accordingly, it appeared that proangiogenic effect of morphine and nicotine is dose and phase dependent (40–47). Compelling evidences indicated that NO is a critical mediator of angiogenesis. And NO has manifold effects on angiogenic processes. NO as a proliferative and anti-apoptotic factor served as an endothelial survival (48), proliferation factor (49). There is a substantial evidence that showed that effective angiogenesis required the synthesis of bioactive endothelium-derived NO. Firstly, a number of angiogenic factors up regulate the endothelial expression of NO synthase (NOS) and stimulate the release of endothelium-derived NO. Vascular endothelial growth factor (VEGF) augmented the endothelial expression of NOS, and stimulated the biosynthesis of NO from cultured human umbilical venous endothelial cells (50). The serum NO concentrations in 2K1C hypertensive rats were lower than sham-clipped group (51).The decreased serum NO level could be the result of either endothelial dysfunction due to hypertension or adaptation to high blood pressure (52). This and decreased NO bioavailability, which was observed in our study, could possibly explain why cardiovascular risk factors such as hypertension are also considered risk factor for atherosclerosis. Reduced NO bioavailability in hypertensive subjects may result from lower NO production such as deficiency in L-arginine/BH4 (53) or increased NO degradation due to higher superoxide anion generation or reduced level of antioxidant (54).Herein, in this study, we did not find any difference in serum NO concentration between these pre-addict and post-addict hypertensive rats surprisingly. That withdrawal of addiction did not alter serum NO concentration. Recent studies proposed the presence of specific opioid receptors, including MO on endothelial cells (20). Morphine activats NO production in the vascular endothelium via attachment to MOR and further leads to vasodilatation (20). Opioids also promote cell proliferation (55) and angiogenesis induced by the endothelial cell specific growth and survival factor, vascular permeability factor/VEGF (56). The chronic exposure to nicotine induces VED by decreasing the generation and bioavailability of NO (16) and elevating the level of asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of endothelial NOS (57). Furthermore, nicotine down-regulates endothelial NOS, an enzyme involved in the generation of NO, decreases endothelium dependent vasodilatation and induces VED and subsequently atherosclerosis (58). Such findings support the idea that endothelial dysfunction and decreased NO generation in coronary artery disease may play the crucial inhibitory role in the response to antigenic agents (59). In conclusion, it seems that changes in blood pressure in morphine and nicotine addicted hypertensive rats are reversible by withdrawal. That probably suggested that in addition, other mechanisms could be involved in reversal of blood pressure to pre-addict level. Lower coronary angiogenesis during addiction and even after withdrawal in our study may propose that possible endothelial dysfunction due to co-administration of morphine and nicotine is reminded even post-withdrawal. It shows the importance of addiction treatment of morphine and nicotine in hypertension in clinical condition.

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