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Effect of Amlodipine and its Combination with L-arginine on Rat Ovarium

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ABSTRACT

Amlodipine is a calcium channel blocker used extensively as an antihypertensive agent in female population. It is reported to possess few pleotropic effects including nitric oxide production. However, its combined effect with L-arginine (a well known nitric oxide donor) on reproductive system is not yet reported. In the present study, experimental rats were divided into three groups and treated with normal saline, amlodipine and Amlodipine + L-arginine respectievely. The test compounds were administered for a period of 30 days followed by which the ovarian tissues were subjected to assay of antioxidant status and histopathology, whereas nitric oxide was estimated in the serum. Amlodipine treated group showed non-significant increase in nitric oxide production but did not demonstrate adverse effects on antioxidant status, oestrous cyclicity and histological structure. However, amlodipine + L-arginine combination significantly ($P<0.05$) elevated lipid peroxidation, SOD and nitric oxide, and significantly ($P<0.05$) decreased GSH concentration in the ovaries. Also, the length of oestrous cyclicity was significantly ($P<0.05$) increased with prolonged diestrous. The histology of the ovaries revealed increased degeneration of follicles and atresia coupled with vasodilation. Thus amlodipine and L-arginine combination adversely affected the ovaries, which could be attributed to the enhanced production of nitric oxide.

Keywords: Amlodipine, L-arginine, nitric oxide, ovary, rats

Amlodipine is a dihydropyridine (DHP) calcium channel blocker indicated in hypertension either as solo therapy or in combination with other antihypertensive agents. It acts through blockade of L-type calcium channels, thus reducing intracellular calcium levels (Haria *et al*., 1995; Opie *et al.,* 2012). Amlodipine is widely prescribed in female population even during pregnancy and lactation (Duley *et al*., 2006; Abalos *et al*., 2007). Although it is reported to cause reproductive toxicity (Lamano *et al*. 2000; Latif *et al*. 2008; Dominic and Padmaja 2013) and gynaecomastia (Cornes *et al*., 2001; Derkacz*et et al*., 2011) in male population, there is insufficient evidence about its reproductive safety in female population at clinical dose. Amlodipine also possesses pleotropic effect, which includes stimulation of nitric oxide production (Mason *et al*., 2013). Amlodipine produces nitric oxide through inhibition of kininase II and activation of angiotensin II enzyme, further stimulating local kinin production and NO release (Mason *et al*., 2003; Batova *et al*., 2006). L-arginine is a physiological nitric oxide donor producing nitric oxide in the presence of nitric oxide synthase enzyme. Thus, Amlodipine in combination with L-arginine produces an additive effect on nitric oxide production (Moncada and Higgs, 1993). Nitric oxide plays a significant role in ovarian folliculogenesis and its modulation carries considerable impact on the physiological functioning and antioxidant status of the ovaries (Rosselli *et al*., 1998; Matsumi *et al*., 2000). Overproduction of nitric oxide leads to cell injury due to peroxynitrite mediated nitrative stress (Tamanini *et al*., 2003).The present study was performed to investigate the safety of amlodipine alone and in combination with L-arginine on ovaries with special reference to oestrous cyclicity, antioxidant status, nitric oxide production and histopathology.

MATERIALS AND METHODS

Animals and design

Young female rats, 8-10 week old, of the *Sprague-Dawley* strain, were procured from Sanzyme Pvt. Ltd., Hyderabad, India. All the animals were maintained under standard conditions prescribed by CPCSEA. The animals were weighed and kept separately in polypropylene cages and were allowed to acclimatize to the experimental conditions for one week before the commencement of the study. They were kept under the standard hygienic laboratory conditions, providing the standard laboratory animal feed and water *ad libitum*. Prior permission was obtained from the Institutional Animal Ethics Committee before the start of the experiment (IAEC, Approval No.4/2017-SA/16-05-2017).

Chemicals

L-arginine and amlodipine were procured from Himedia, Mumbai and Sisco Research Laboratories Pvt. Ltd., Mumbai, respectively. All the other chemicals used were of analytical grade.

Experimental procedure

The animals were divided into three groups comprising of six animals each. Group I served as normal control and received 1 ml of distilled water throughout the experiment. Group II served as amlodipine-treated group and received amlodipine at the rate of 1 mg/kg/day (corresponding to clinical dose of 10 mg). Group III served as amlodipine + L-arginine-treated group, wherein amlodipine was administered orally at the rate of 1 mg/kg and L-arginine was added at the rate of 1.8 % in drinking water. The test compounds were administered for a period of 30 days, followed by which the animals were humanely sacrificed and subjected to biochemical analysis and histopathology.

Oestrous cycle evaluation

The oestrous cycle is the one of the prime targets affected in a hypothalamic-pituitary-ovarian reproductive axis malfunction and hence analysis of oestrous cyclicity carries immense importance in determination of ovarian functioning (Goldman, 2007). In the present study, seven consecutive oestrous cycles were measured in all the three groups by vaginal smear test (Cooper *et al*., 1992; Zarrow, 2012). Vaginal smear was taken early in the morning on a regular basis by injecting few drops of normal saline (0.9% NaCl) into the vagina followed by collection with a micro-tipped Pasteur pipette on a clean plain glass slide. The cells in the smear were observed under light microscope.

Antioxidant analysis in ovarian tissue

At the end of the experiment, ovarian tissues were collected to assay reduced glutathione (GSH), super oxide dismutase (SOD) and lipid peroxidation. Ovarian tissues were homogenized in 10 mM Tris HCl buffer at pH 7.1 followed by centrifugation at 12000 g for 10 min. The supernatant was used for further analysis. The estimation of SOD involved inhibition of superoxide-dependent reduction of tetrazolium dye MTT to formazan (Madesh and Balsubramanian, 1998). Quantitative estimation of GSH was performed by interaction of reduced GSH with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) followed by measurement of absorbance at 412 nm (Moron *et al.,* 1979), whereas lipid peroxidation was estimated by the method described by Balasubramanian *et al*., 1988

Estimation of nitric oxide

Estimation of Nitric oxide was performed as per Miranda *et al.,* 2001. Serum and ethanol were mixed in 1:1 ratio and centrifuged at 13000 rpm for 20 minutes. The supernatant was separated and used for further assay. A 100 ul of supernatant was added to equal quantity of vanadium chloride and Griess reagent followed by immediate incubation at 37 ˚C for 20-30 minutes. The absorbance was measured at 540 nm. Sodium nitrite (0.5-25 µM) was used as standard for Calibration. The values obtained were expressed as units of nitrate per litre.

Histopathology

On termination of the study, the ovarian tissues were collected and fixed in 10% neutral buffered formalin for histopathological analysis. The samples were processed and stained with haematoxylin and eosin (H and E).

Statistical analysis

The data obtained from the present study were analyzed

by using the one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test using the Graph Pad prism software (Graph Pad Prism, version 7). Values were considered significant if P<0.05.

RESULTS

Impact of Amlodipine and its combination with L-arginine on length of oestrous cycle

Amlodipine treated group demonstrated normal oestrous cycling with a mean length of 4.205±0.02 as compared to normal control which had a mean length of 4.235±0.02, whereas Amlodipine + L-arginine treated group showed significant (P<0.05) increase in the length of oestrous cycle with a mean length of 6.007±0.03 compared to control group (Fig. 1).

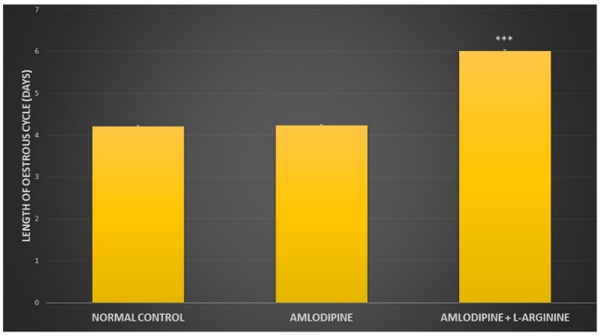

Fig. 1: Length of oestrous cycle in normal control and treated groups

Results are represented as Mean ± SEM. Asterisk (***) indicate significant p-values at P≤0.05

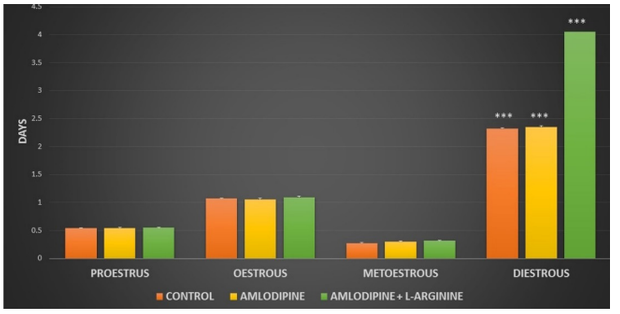

Fig. 2: Length of various phases of oestrous cycle in normal control and treated groups

Results are represented as Mean ± SEM. Asterisk (***) indicate significant p-values at P≤0.05

The prolongation was primarily due to significant (P<0.05) increase of diestrous phase in the Amlodipine + L-arginine treated group. The mean length of diestrous phase in normal control group was 2.32±0.01 whereas it was 2.35±0.02 and 4.05±0.01 in Amlodipine and Amlodipine + L-arginine treated groups respectively (Fig. 2).

Effect of Amlodipine and amlodipine + L-arginine on antioxidant status

Amlodipine did not significantly affect the antioxidant status of the rats with a mean SOD concentration of 8.162±0.31, GSH concentration of 5.98±0.162 and lipid peroxidation level of 13.81±0.32. The mean concentration of SOD, GSH and Lipid peroxidation in normal control animals were 8.308±0.3, 5.935±0.165 and 13.14±0.34 respectively. However, combination of Amlodipine + L-arginine significantly (P<0.05) increased the lipid peroxidation and decreased (P<0.05) the superoxide dismutase and GSH concentration in the ovarian tissues (Fig. 3). The mean concentration of SOD and GSH decreased to 4.882± 0.3 and 2.37±0.33 respectively whereas the lipid peroxidation increased to 18.84±0.34 as compared to the normal control group.

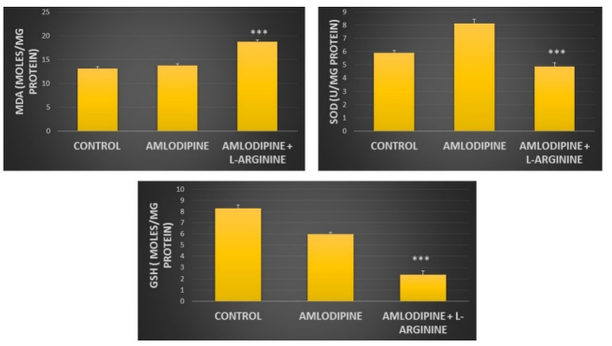

Fig. 3: Antioxidant parameters (SOD and GSH) and lipid peroxidation (MDA) levels in normal control and treated groups

Results are represented as Mean ± SEM. Asterisk (***) indicate significant p-values at P≤0.05

Effect of Amlodipine and amlodipine + L-arginine on nitric oxide levels in serum

Amlodipine treated group demonstrated slight increase in the nitric oxide concentration with a mean increase of 3.915±0.12 as compared to normal control group which had a mean concentration of 3.55±0.12. However,

amlodipine + L-arginine treated group demonstrated significant (P<0.05) increase in nitric oxide concentration with a mean concentration of 9.152±0.11 as compared to normal control group (Fig. 4)

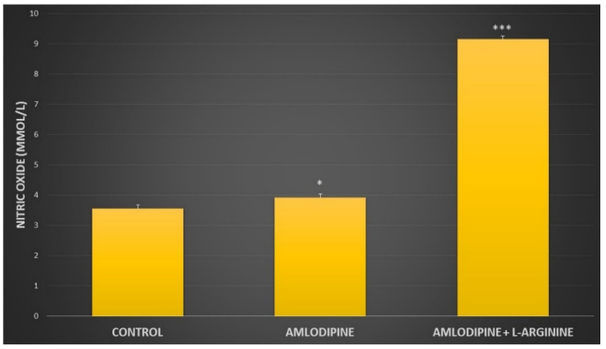

Fig. 4: Nitric oxide levels in normal control and treated groups

Results are represented as Mean ± SEM. Asterisk (***) indicate significant p-values at P≤0.05

Histopathological changes in amlodipine and amlodipine + L-arginine treated rats

Ovarian histology of control group showed normal architecture. Amlodipine treated animals did not reveal any pathological changes microscopically.

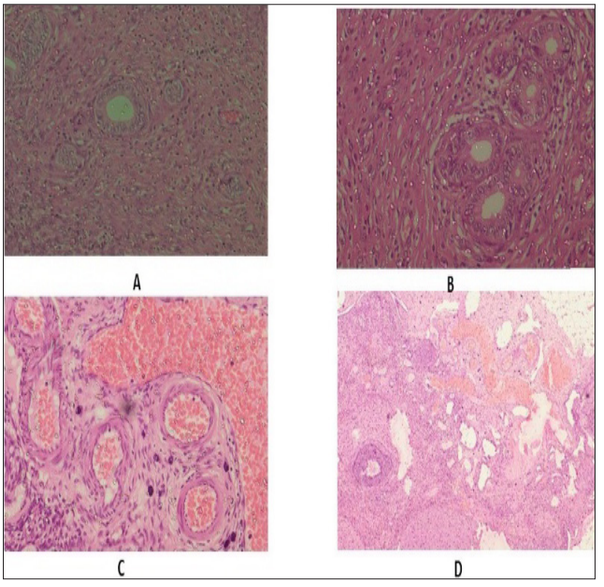

Fig. 5: Photomicrograph of ovary of normal control and treated groups (H&E × 20 ; 300 dpi)

However the animals treated with Amlodipine + L-arginine combination revealed vacuolar degeneration of granulosa cells with dense pyknotic nuclei, degeneration of primary and secondary follicles, vasodilation of capillaries, and increased follicular atresia (Fig. 5).

DISCUSSION

Amlodipine and nitric oxide donors are commonly used antihypertensive agents. Both the compounds exert their vasodilatory effect through different, however, convergent mechanisms. Amlodipine is a calcium channel blocker that inhibits L-type calcium channels leading to reduced intracellular calcium levels (Haria and wagstaff, 1995). Amlodipine also produces nitric oxide through mechanisms other than nitric oxide synthase pathway (Stepien *et al*., 2002; Mason *et al*., 2003; Mahajan *et al*., 2007). A few pre-clinical studies have reported role of Amlodipine in teratogenicity and adverse effects on male reproduction leading to male infertility and gynaecomastia but its clinical relevance is yet to be established (Tranquilli and Giannubilo, 2009; Dominic and Padmaja, 2013). Uterine toxicity associated with amlodipine has been previously observed, but at a dose twice as that of the clinically prescribed dose (Salman *et al*., 2011). In the present study, Amlodipine did not cause any significant changes in the length of the oestrous cycle and antioxidant status. Although amlodipine slightly increased the nitric oxide production as compared to control group, the animals did not undergo oxidative stress. This could be attributed to its pleotropic effects on the ovaries (Mahajan *et al*., 2007; Halici *et al*., 2008; Mason *et al*., 2013). Amlodipine did not demonstrate any pathological change in the ovaries leading to the conclusion that amlodipine at a clinical dose does not pose concern to the ovarian functioning.

On the other hand, combination of Amlodipine and L-arginine lead to significant disruption of oestrous cyclicity and prolongation of diestrous phase. The combination also demonstrated significant increase in oxidative stress through elevated lipid peroxidation coupled by decrease in GSH and superoxide dismutase levels. These changes could be attributed to the exacerbated production of nitric oxide caused by the additive effect of Amlodipine and L-arginine leading to peroxynitrite mediated nitrative stress (Dave *et al*., 1997; Xia *et al.,* 2010). As nitric oxide has a significant role in oestrous cyclicity and ovulation patterns, disruption of oestrous cyclicity could be the result of

enhanced production of nitric oxide (Nemade *et al*., 2002). The combination caused histopathological alterations that included vacuolar degeneration of granulosa cells, dense pyknotic nuclei, degeneration of primary and secondary follicles, vasodilation of capillaries, and increased atresia. These results corroborated to previously reported studies reporting toxicity produced by over production of nitric oxide on ovarian folliculogenesis (Yamauchi *et al.,* 1997; Hassani *et al*., 2012; Dubey *et al.,* 2016).

In conclusion, with respect to safety of amlodipine on ovaries, further studies assessing the impact of amlodipine on hormonal parameters and effect of the drug on pre- and post-natal stages of reproduction are essential to arrive at a definite conclusion regarding the reproductive toxicity of amlodipine. Also, since amlodipine as an antihypertensive drug is consumed for a longer duration, chronic studies are required to determine its long term safety. Combination of vasodilating agents such as amlodipine and L-arginine should be carefully studied prior to clinical use in women population. Also, the molecular mechanism underlying these effects should be explored with the intention of delivering safe drug combinations to the hypertensive female population.

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CONFLICT OF INTEREST

All authors have no financial or personal relationship with organizations or people that could influence or bias the study.

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