IJPSR (2015), Vol. 6, Issue 3 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 July, 2014; received in revised form, 28 September, 2014; accepted, 01 December, 2014; published 01 March, 2015

INFUSION OF ADRENOMEDULLIN-2 ANTAGONIST INDUCES APOPTOSIS AND CHANGES m-RNA EXPRESSION LEVELS OF ADM2 RECEPTORS IN PLACENTA OF PREGNANT RATS DURING LATE GESTATION AND CAUSES FETOPLACENTAL GROWTH RESTRICTION

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Keywords:

Adrenomedullin2, osmotic mini pumps, labyrinth zone, Progesterone & Estrogen, Caspase-3

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ABSTRACT: Adrenomedullin-2 or Intermedin (ADM2/IMD) is newly identified novel hormone belongs to peptide family like AM, calcitonin, CGRP, amylin and it is widely distributed in various tissues including ovaries, uterus & placenta. The aim of present study was to investigate the role of endogenous ADM2 in fetoplacental development in pregnant rats during late gestation period through infusion of ADM2 antagonist. For this study we have infused ADM2 antagonist ($ADM2_{17-47}$) at 50 and 200 µg / rat / day exposed continuously by inserting osmotic mini pumps to pregnant wistar rats on gestational day 18 and were sacrificed on gestational day 22.The results showed that infusion of ADM2 antagonist significantly decreases ($P<0.05$) the total sac weight, fetoplacental weights, length of the fetus and also the serum progesterone & estrogen levels. High molecular DNA fragmentation was observed in $ADM2_{17-47}$ treated placental tissues and in immunohistopathological studies apoptotic cells (pyknosis & karyorrhexis cells) and active caspase-3 positive immunoreactivity cells were observed in labyrinth zone of placental tissues. In western blot analysis the levels of caspase-3 was significantly increased in $ADM2_{17-47}$ treated rats than compared to controls. Furthermore, antagonism of ADM2 antagonist significantly decreased ($P<0.05$) the mRNA expression levels of ADM2 receptors CRLR, RAMP1, and RAMP3 but not in RAMP2. Finally we conclude that ADM2 antagonist acts as a non-competitive inhibitor for endogenous ADM2 and inhabited the actions of ADM2 in fetoplacental development and causes fetal growth restriction.

INTRODUCTION: The placenta is a unique, autonomous and transient organ which is responsible for maternal-fetal exchanges and maternal tolerance of fetopaternal antigens. In some species, the placenta performs hormonal functions required for the maintenance of gestation and fetal well-being [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(3).1137-49

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(3).1137-49

The placenta also recognized as a source of various biologically active peptides which plays an important role in the maintenance of pregnancy and in maternal homeostasis during pregnancy and it is a remarkable organ not only provides the conduit for alimentation, gas exchange, and excretion the fetus, it also is a major endocrine organ, producing a plethora of protein (including cytokines and growth factors) steroid hormones and peptides which it secretes in large quantities primarily into the maternal circulation [2].

In this connection calcitonin/calcitonin gene-related peptide (CGRP), Adrenomedullin (AM) and amylin are first identified and isolated from teleosts and

these are endocrine regulators for the adaptation and regulation of homeostasis in vertebrates. This distinctive cluster of peptides shares a preserved tertiary structure with associate N-terminal disulfide-bridged ring and these peptides signal through two closely related type II GPCRs and three unique receptor activity-modifying proteins (RAMP). Recently, based on the analysis of multiple vertebrate genomes a novel calcitonin/CGRP family related peptide was identified and named as Intermedin (IMD) due to its abundant expression in the intermediate lobe of the anterior pituitary [3, 4].

The peptide Intermedin (IMD) is a 47 amino acid peptide and it also referred as adrenomedullin2 (ADM2) because intermedin is closely related to adrenomedullin (AM). IMD/ADM2 discovered independently by the two research groups in 2004. Roh et al.[5] identified this novel peptide gene encoding a prepro- peptide of 146– 150 amino acids and the C-terminal region of this prepro-peptide encompassed a putative 47 amino-acid peptide sharing ~28% sequence identity with AM and < 20% with CGRP and predicted to possess the common structural features characteristic of members of the peptide super family [5].

Takei et al.[6] examined mammalian complementary DNA libraries for potential mammalian homologues of five orthologues of AM (AM1–AM5) which they had identified in puffer fish. Using this approach, they also identified a 146–150 amino-acid mammalian prepro-peptide which yielded a mature peptide containing 47 amino acids; this displayed ~ 30% sequence homology to AM and > 70% homology to the puffer fish and this novel mammalian peptide was named adrenomedullin-2 (ADM2)[5,6].

AM2/IMD has vasodilatory and hypotensive actions that were just like or firmer than those of ADM and CALCB and it had been distributed in cells and tissues overlap with AM. Compared with AM, ADM2 is a smaller amount cosmopolitan in mammals &usually this amide is found within the brain, pituitary, heart, kidney, digestive tract, plasma, pancreas, lung, spleen thymus, uterus, ovaries and placenta [6, 7]. *Taylor et al. and Samson et al.*[8] reported that AM2/IMD may play a role in the regulation of prolactin release during reproduction in females and also peripheral injections of AM2/IMD elevates the levels of oxytocin and vasopressin in plasma [8, 9, 10].

Generally the calcitonin/CGRP family peptides signal through two closely related type II GPCRs. Calcitonin signals through the calcitonin receptor whereas efficient signaling by amylin requires the formation of a receptor complex of the calcitonin receptor and selected receptor activity-modifying proteins (RAMPs). In contrast, adrenomedullin and CGRP tranduce there signals mainly through the CRLR/RAMP receptors consist of CRLR and one of the three RAMPs. Whereas CGRP primarily signals through the CRLR/RAMP1 receptor, adrenomedullin has a greater efficacy on the CRLR/RAMP2 and CRLR/RAMP3 receptors and the newly identified AM2 binds nonselectively to all three CRLR/RAMP complexes but it has a high potency with CRLR/RAMP1 and CRLR/RAMP3 receptors [5, 11, 12].

Madhu chauhan and Uma yallampalli et al.[13] reported that in pregnant rats ADM2 is elevated highly in rat mesenteric artery and the plasma level of immunoreactive ADM2 is increased during pregnancy, the author suggests that ADM2 play a role in local control of vascular tone as an autocrine or paracrine regulator. ADM2 mRNA expression was detected in human placental villi & in first-trimester cells and also it was identified that throughout the pregnancy the ADM2 has a role in regulation of trophoblast invasion and migration [14].

Further more in another report it showed that infusion of ADM2/IMD_{17-47} antagonist during mid gestation in rats causes feto-placental growth restriction and significant decline in the expression of NOS 1/2/3 in rat placenta on day 15 of gestation [15].

Therefore, findings of the above studies it shows that IMD has many actions similar to those of AM and CGRP and this novel peptide has great role in female reproductive function during pregnancy. Based on this the aim of the present study was to examine whether $ADM2_{17-47}$ antagonist causes apoptosis and also to asses changes in the mRNA expression levels of ADM2 receptors (CRLR,

RAMP1,RAMP2& RAMP3) in placental tissues of pregnant rats during late gestation period.

MATERIALA AND METHODS:

Maintenance of Experimental Animals:

Healthy female rats of Wistar strain were purchased from authorized vendor (M/S Raghavendra Enterprises, Bangalore, India). All rats were housed in polypropylene cages (18" 10"x 8") lined with sterilized paddy husk, and provided filtered tap water and rat food ad libitum in an air-conditioned environment (25±2°C) with a 12-h light and 12-in dark cycle. All animal experiments were approved by the Animal Experimental Ethical Committee, the institute of SPMVV, Tirupati.

Experimental Design:

Female wistar rats (Rattus norvergicus) three months old, weighing 200g ± 300 g were used for the experiment .The status of estrous cycle stages were determined every morning between 8:00 and 9:00 a.m by collecting of vaginal secretion with a plastic pipette filled with 10 µL of normal saline(NaCl 0.9%) by inserting the tip into the rat vagina[16]. One drop of vaginal fluid was placed on glass slides the unstained material was observed under a light microscope [16, 17]. Two females of pro-estrous stage were paired with a male overnight and the next morning, males were removed and females were assessed for the presence of sperm in the vaginal flush. Animals with positive sperm in the flushes are designated as day 1 of gestation. Six pregnant rats were used in each experimental group.

Infusion process and Collecting placentas and fetuses at late-gestation:

On gestational day 18 the osmotic minipumps (model 2ML1; 10 µl/h; Alzet minipumps, U.A, CA) were inserted subcutaneously into the dorsum of pregnant rats while the animals were under anesthesia. Anesthesia consisted of a combination of ketamine (45 mg/kg) and xylazine (5mg/kg S.V. Veterinary college of Science, TPT). The minipumps were filled with saline alone infused to control rats and with saline containing two different concentrations of $ADM2_{17-47}$ at 50 µg /rat/day and 200 µg /rat /day infused to pregnant rats, the dosage was selected based on earlier findings [15]. To assess steroid hormones levels in serum, blood was collected by cardiac puncture before sacrifice the rats, then all the pregnant rats were sacrificed on gestational day 22 using a CO_2 inhalation chamber. The uterus was exteriorized and weighed, and embryonic sacs were separated from the uterus and also weighed. Placenta and fetal membranes were separated and weighed individually, then fetoplacental weights & lengths of the fetus was measured. Amniotic fluid volume was derived by subtracting placental, fetal membrane, and fetal weights from total sac weight. In addition the uteroplacental tissues were cleaned, frozen immediately in liquid nitrogen for histopathology, DNA fragmentation and immunohistochemistry and some uteroplacental tissues are stored at -80^{o} C until use for western blot and RT-PCR analysis.

Radioimmunoassay for serum steroid hormone levels:

Blood was collected through cardiac puncture follwed by serum extraction for the measurement of 17 β- estradiol and progesterone. RIA was performed by using radioimmunology assay kit accordance with manufacturer's instructions (Merck Millipore, Bangalore, India).

Histopathological examination:

For this portion of the study, placentas were collected from rats infused with 50 and 200 µg/day of $ADM2_{17-47}$ along with control group. Immediately the collected placentas were placed in fixative solution (10% formaldehyde) later the placental tissue block was dehydrated with different series of isopropyl alcohol concentrations then the placenta tissues are embedded in paraffin and sectioned the tissues into 5- µm thickness finally tissue sections are transferred on to microscopic slide and were stained with hematoxylin and eosin and then studied by light microscope (OLYPHUS). Appropriate photographs were taken with a digital camera (COOLPIX, Nikon).

DNA fragmentation analysis:

The DNA was extracted from placental tissues using phenol / chloroform (1:1) method .(Placental tissues were homogenized in a lysis buffer (50mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% sodium – N-laurolysarcosinate & Fragmentation of DNA was qualified by the diphenylamine method.) Briefly,

the lysate was centrifuged at 13000xg at 4^0C for 20 min to separate intact and fragmented chromatin. Each fraction was treated with 0.2 ml of 6% perchloric acid (PCA) for 30 min at 4^0C. Precipated functions were treated with 50 µl of 6% PCA at 70^0C for 20 min. 0.1ml of diphenylamine solution (1.5% diphenylamine, 1.5% sulfuric acid, 0.01% acetaldehyde in acetic acid) was added to the samples and these were allowed to stand overnight at 30^0C.

The samples were then determined spectrophotometrically at 600 mm, and the percentage of fragmented DNA was calculated from the amount of DNA found in the supernatant. Ladder-like profiles of electrophoresed DNA samples were analyzed. Finally homogenates were incubated with 1 mg / ml of proteinase K for 30 min and subsequently with 1mg / ml of RNase for 30 min at 50^0C. The DNA samples thus obtained (1.5 from 5 µg of total DNA) were subjected to 2% agarose gel electrophoresis at 100 V using a running buffer containing 90 mMTris-HCl (pH 8.0), 90 mM boric acid and 2 mM EDTA. After electrophoresis, gels were stained with 0.1µg / ml ethidium bromide and observed under ultraviolet light.

Immunohistochemistry:

After deparaffinization of tissue sections, antigen retrieval was performed by heating in 10 mM citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. After blocking with 5% normal rabbit serum, sections were incubated with the primary antibody for caspase-3 (polyclonal antibody at 1:100 dilution) at 48^O_C overnight. They were rinsed in PBS and incubated with biotinylated goat anti-rabbit immunoglobulin IgG, followed by avidin-biotinperoxidase solution. Next, DAB with 0.003% H_2O_2 in PBS was added to each slide. The tissues were lightly counterstained with hematoxylin and examined by light microscopy [18].

Western blot analysis:

Equal amounts of protein (40 µg) were separated by various appropriate concentrations of SDS-PAGE: 12% for Bcl-2 and 15% for caspase-3. Gels containing the SDS-PAGE-separated proteins were equilibrated in transfer buffer (25 mM Tris, pH 8.3, 190 mM glycine, 0.05% SDS, and 20% methanol) and were electro transferred to nitrocellulose membranes. Membranes were blocked with TTBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 1 h and were washed with TTBS buffer. For the detection of apoptotic and antiapoptotic proteins, nitrocellulose membranes were incubated in the antibodies to Bcl-2 and caspase-3. After exposure to horseradish peroxidase-conjugated anti-rabbit IgG (caspase-3 and Bcl-2) secondary antibodies (diluted 2000-fold to 5000-fold) for 1 h, blots were washed and developed by enhanced chemiluminescence.

Each blot was stripped with 100 mM glycine, pH 2.3, and was reprobed with β-tubulin to normalize for any variations incorporated in protein loading. Densities of each protein of interest were expressed as a ratio to that of β-tubulin [18].

Isolation of Total RNA from placental tissues:

To determine whether $ADM2_{17–47}$ infusion alters the expression of ADM2 receptors in uteroplacental tissues for that total RNA was extracted using TRIzol reagent method [19]. Briefly, TRIzol tissue homogenate was spin at 12,000X 5 for 5 minutes at 4^oc, clear TRIzol lysate supernatant was taken then 0.2ml chloroform was added to 0.8ml TRIzol lysate and centrifuged at 12,000 X g for 15 minutes at 4^oc to perform phase separation. After centrifugation the upper aqueous layer was taken and 75% ethanol was added to precipitate RNA. The RNA precipitate was dissolved in 20 µl of ribonuclease (RNase)-free water containing deoxyribonuclease (DNase) I buffer and 2 units of amplification grade DNase I to avoid DNA contamination. The DNase I will be removed by phenol chloroform extraction. The isolated RNA was dissolved in RNase-free water and it was stored at –80°C until use .The quality &quantity of the RNA were assessed at A 260/280, and all samples showed absorbency ratios ranging between 1.6 and 2.0.

Reverse Transcription & Polymerase Chain Reaction (RT-PCR):

The first-strand cDNA was synthesized by reverse transcription (RT) for that in a 20-µl reaction volume containing, PCR buffer, 2 µg of total RNA were mixed with 3.0 nmol of random primer, 1mM

dNTP solution and 10 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase in the presence of 5 U of RNase inhibitor and placed in a thermal cycler for one cycle at 28^{o}C for 15 min, 42^{o}C for 30 min, 99^{o} C for 5 min, and 4^{o}C for 5 min. The polymerase chain reactions was initiated for CRLR, RAMP1, RAMP2 and RAMP3 by the specific primer set designed based on the published

DNA sequence [20, 21, 22]. Briefly, 2 µl of cDNA will be mixed with a PCR mixture containing 2.5 mM $MgCl_2$, 1x of 10x PCR buffer, 5 U/100 µl of 1 mM dNTP mixture, and 0.2 µM of the following gene-specific primers: Primer sequences are as follows: CRLR:forward,5'TGCTCTGTGAAGGCATTTA3', andreverse,5'CAGAATTGCTTGAACCTCTC3';RAMP1:forward,5'GAGACGCTGTGGGTGACTG3', and reverse, 5'TCGGCTACTCTGGACTCCTG3'; RAMP2:forward,5'GCTGTTACTGCTGCTGTTGC3',andreverse,5'GTCTGCCTCGTACTCCAAGC3';andRAMP3:forward,5'CTTCTCCCTCTGTTGCTGCT3',andreverse,5'GTCCTGTCCACAGTGCAGTT3'.andβactin:forward,5'GTCGACAACGGCTCCGGCA3'andreverse,5'GTCAGGTCCCGGCCAGCCA 3'. β- actin , were derived from rat β- actin cDNA sequence [23].

The PCRs were carried out at the following conditions: an initial denaturation step at 95 ^{o}C for 7 min, followed by 35 cycles of 30 sec at 95^{o}C, 1 min at 60^{o}C, and 30 sec at 72^{o}C. Reaction conditions for PCR of RAMP2 and RAMP3 were similar except that the annealing temperature was 63^{o}C. All the reactions were terminated by a 7-min long elongation step at 72 ^{o}C.

Electrophoresis and Gel Imaging:

The PCR products were loaded on 1.8% agarose gel containing 0.5 µg/ml ethidium bromide and run in 0.5% Tris-borate buffer at 100 V for 2 h. Gels were placed on a UV light box, imaged, and then analyzed with Sigma gel system. Density-gradient measurements were performed using the image J software (N1H). The integrated band density of product bonds was calculated in arbitrary units.

Data Analysis:

The given data were expressed as mean ± SD. The results obtained were analyzed and compared by one-way analysis of variance (ANOVA) for comparing various doses of ADM2 antagonist. Student t-test was performed for statistical comparison between treated and controls. *P* values less than 0.05 were considered statistically significant.

RESULTS:

Effect of ADM2 $_{17-47}$ on fetoplacental Weights & lengths:

In this portion of the study, we evaluated the role of ADM2 on fetoplacental growth rate during pregnancy. The detrimental effects of ADM2 antagonist shown that there is significant decrease in total sac weights, fetoplacental weights and also there is a significant decrease in fetal lengths (*P< 0.05*; **Table: 1** and **Figure: 1**). These reductions in fetoplacental weights & lengths were more substantial with ADM2 $_{17-47\ at}$ 200 µg /rat /day compared with 50 µg /rat /day. There was no significant differences found in amniotic fluid, litter size and uterine weights compared to saline treated controls. The fetus & placenta were collected from control rat and **(B&D)** the other fetus & placenta on the right side were collected from ADM2 antagonist treated rats with 200 µg /rat /day on day 18 of gestation. Infusion of ADM2 antagonist significantly decreased (*P< 0.005*) the fetoplacental weights & lengths compared to controls.

TABLE 1: TOTAL SAC WEIGHTS, UTERUS WEIGHTS, FETOPLACENTAL WEIGHTS & LENGTHS OF ADM2$_{17-47}$ INFUSED PREGNANT RATS COMPARED WITH SALINE TREATED CONTROL PREGNANT RATS.

Groups/Parameters	Control	ADM2 50µg/rat/day	ADM2 200 µg/rat/day
Pregnant rats per group(n)	06	06	06
Litter size (n)	14 ±0.88	13 ± 0.87	14 ±0.89
Total sac weight (g)	15.6±1.27	15.4±1.30*	48.18±0.40**
Uterus weight (g)	3.77±0.01	3.76±0.03	3.75±0.03
Placental weight (g)	0.48 ± 0.01	0.40 ± 0.04*	0.32 ± 0.08**
Fetal weight (g)	4.92 ± 0.08	3.92 ± 0.13*	3.54 ± 0.15**
Fetal length(cm)	4.36 ± 0.05	4.02 ± 0.08*	3.64 ± 0.15**
Amniotic fluid (g)	2.84±0.06	2.83±0.04	2.84±0.05

Values are expressed as the mean± S.D: **P< 0.005*, ***P< 0.001*

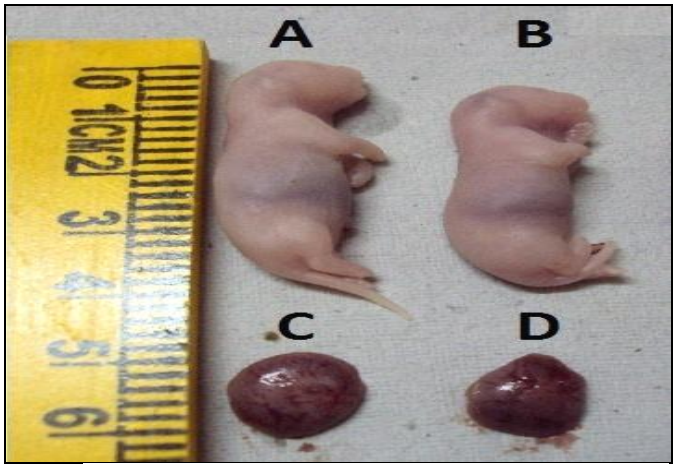

FIG1: PHOTOGRAPHS OF FETUSES & PLACENTAS COLLECTED ON DAY 22 OF GESTATION. (A& C)

Effect of ADM2 $_{17-47}$ on sex setiod hormone levels in pregnant rats:

Progesterone and estrogen are two important sex steroid hormones maintain the pregnancy throughout gestation period. In this study we assess if the ADM2 $_{17-47}$ alters the synthesis of estrogens and progesterone during pregnancy, we measured 17β estradiol and progesterone in the serum from the control and ADM2 $_{17-47}$ treated rat on day 22 of gestation. As shown in **Fig. 2A & 2B** infusion of ADM2 $_{17-47}$ to pregnant rats from gestational day 18 - 22 caused a significant decline ($P < 0.05$) in the levels of both 17β-estradiol and progesterone compared to controls.

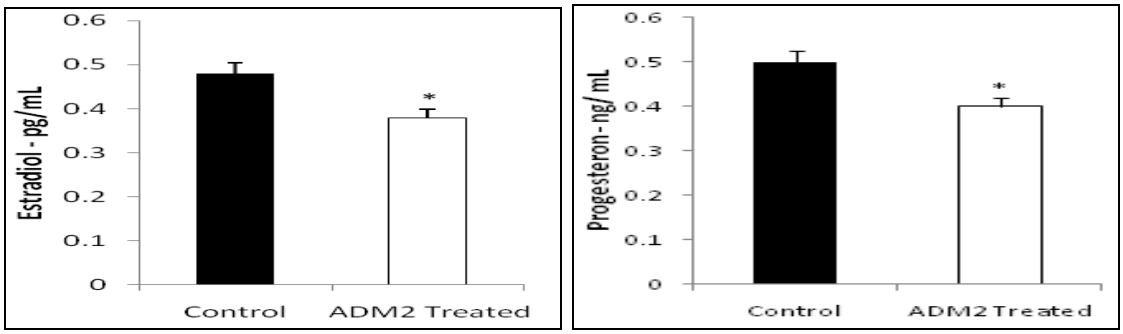

FIG. 2 RIA ANALYSIS OF REPRODUCTIVE HORMONES IN ADM2$_{17-47}$ TREATED PREGNANT RATS: (A&B)

17β-estradiol and Progesterone . Serum samples are collected on gestational day 22 in rats infused with ADM2 $_{17-47}$ (50 & 200 µg/day) or vehicle alone. Asterisks (*) indicates significantly (*P< 0.005*) different compared with the controls

DNA Fragmentation analysis:

DNA fragmentation is a hallmark for apoptosis; in the present study we evaluated the role of ADM2 in fetoplacental development during rat pregnancy at late gestation. DNA fragmentation analysis was performed in placental tissues of ADM2 antagonist treated pregnant rats and in saline treated pregnant rats. **Fig. 3A and 3B** shows fragmentation in placental tissues in rats receiving the two doses of ADM2 antagonist. The degree of percentage of DNA fragmentation was increased significantly ($P < 0.05$) than those in controls and high molecular DNA fragmentation was observed in placenta. Fragmentation in placental tissues were more substantial with ADM2$_{17–47}$ at 200 µg/day compared with 50 µg/day.

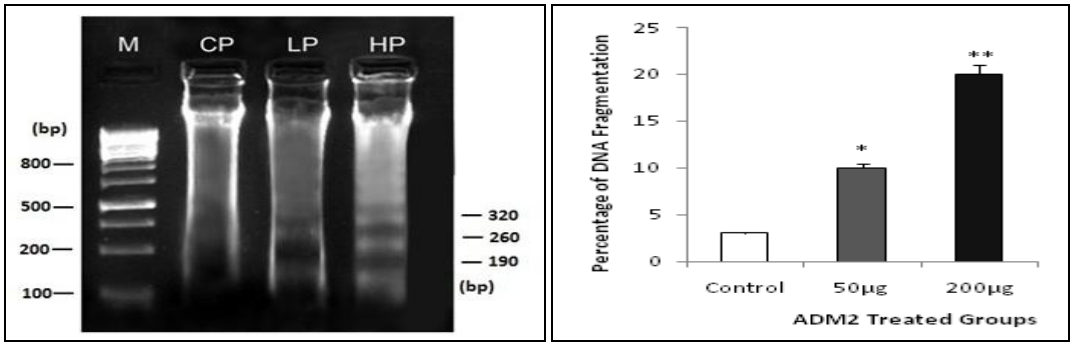

FIG 3: DNA FRAGMENTATION ANALYSIS IN PLACENTAL TISSUES OF ADM2$_{17-47}$ TREATED PREGNANT RATS

M- marker, **CP**- Control Placenta, **LP**- Low dose treated Placenta, **HP**- High dose treated Placenta. 3A, Agarose gel electrophoresis showing high molecular weight DNA fragmentation inplacenta and DNA Ladder showing 190,260 & 320 bp bands of fragmented DNA. **3B**, Quantitation of DNA Fragmentation and percentage of DNA fragmentation compared to controls. Values are expressed as Mean ± SD ($P<0.01$) versus control.

Histopathological observation in placental tissues of ADM2 antagonist treated pregnant rats:

In this study, we examined H&E stained microphotograph of rat placental sections obtained from $ADM2_{17-47}$ treated and vehicle control mothers on gestational day 22. In the treated rats histopathological changes were more prominent in the fetal compartment of placenta (labyrinth & Basal zone) rather than in the maternal compartment (Decidus). In labyrinth zone $ADM2_{17–47}$ infusion caused impairment of labyrinths and exhibited decreased fetal vascular development and in the basal zone, an increase in the number of spongiotrophoblasts and clusters of glycogen cells were detected particularly remarkable at the edge of the basal zone compared to controls (**Fig. 4A & 4B)**. Fetal growth as well as placental weights were substantial lower in $ADM2_{17-47}$ treated rats, we examined for the possibility of the involvement of apoptotic changes in the placental tissues. As shown in the (**Fig. 5A&5B)** the majority of apoptotic cells characterized by pyknosis or karyorrhexis cells are observed in labyrinth zone of placenta.

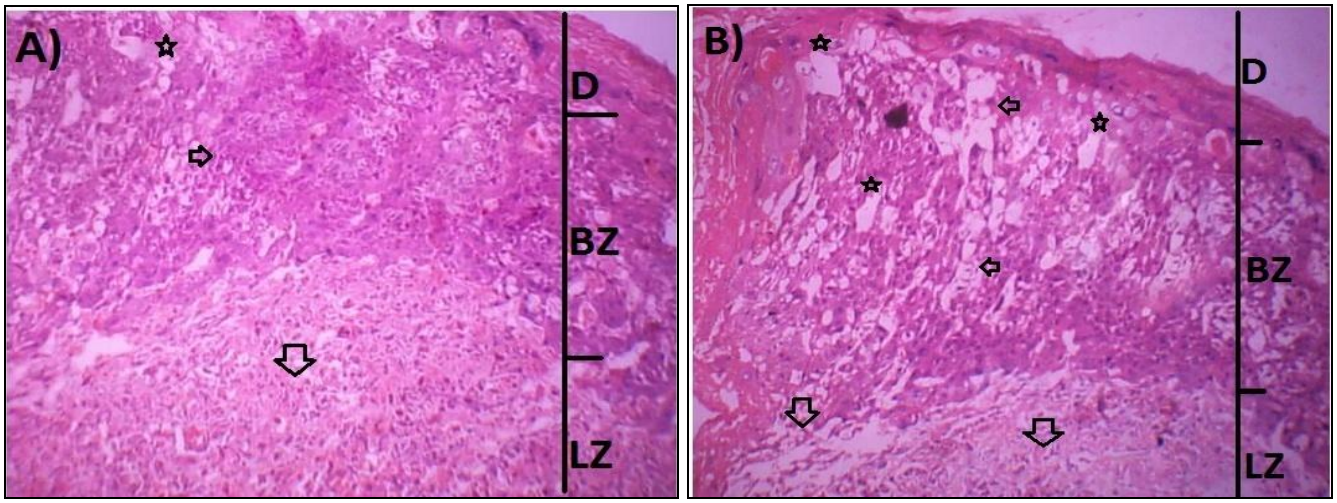

FIG 4: REPRESENTATIVE PHOTOMICROGRAPH OF PLACENTA ON DAY 22 WITH H&E STAIN.

A) Control **B)** ADM217-47 treated placenta. Top bracket (D) indicates normal decidua, middle bracket (BZ) indicates basal zone & lower bracket (LZ) indicates labyrinth zone, star indicates glycogen cells, small arrow indicates spongiotrophoblasts and large arrow for impairment of labyrinths. (X 40 maginification)

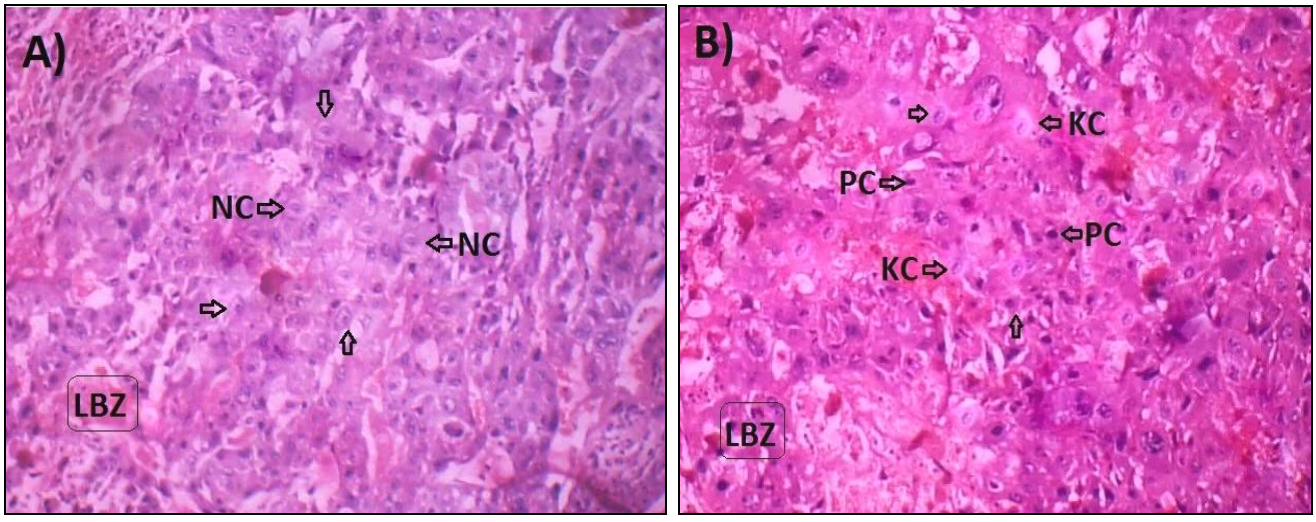

Fig 5: REPRESENTATIVE PHOTOMICROGRAPH OF LABYRINTH ZONE IN PLACENTA ON DAY 22 WITH H&E STAIN.

A) Control; LBZ- Labyrinth zone & NC- Normal cells. **B)** ADM217-47 treated placenta; in labyrinth zone trophoblast apoptotic cells characterized by PC- pyknosis cells & KC- karyorrhexis cells. (X100 magnification).

Observation of immunohistochemical changes in placental tissues of ADM2 antagonist treated pregnant rats:

Immunohistochemical staining studies reveals the presence of active caspase-3 protein in the placental tissues of $ADM2_{17–47}$ treated animals are presented in **Fig. 6A & 6B**. The active caspase-3 protein appeared to be abundant in the placental labyrinth zone of the $ADM2_{17–47}$ -treated rats compared with untreated controls. Similarly, active caspase-3 protein staining was undetectable in untreated controls. Specificity of the staining was confirmed by the absence of staining when primary antibody was omitted in the reaction.

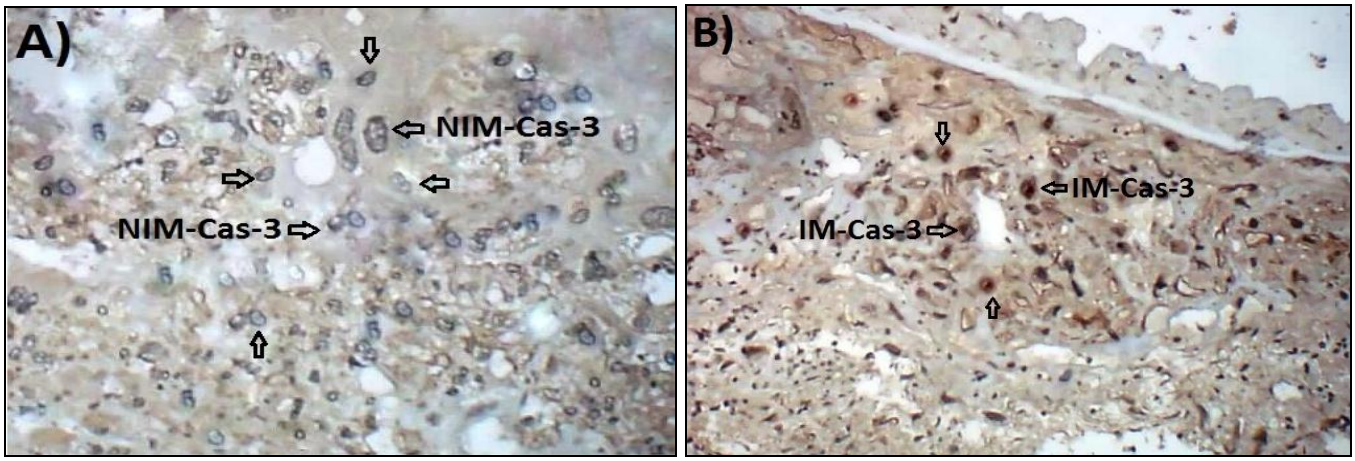

FIG 6: REPRESENTATIVE PHOTOMICROGRAPH OF LABYRINTH & BASAL ZONES OF PLACENTA ON DAY 22 WITH IMMUNOHISTOCHEMICAL STAINING FOR ACTIVE CASPASE-3.

A) Control; The vehicle treated labyrinth & basal zones of placenta showing non immunoreactive caspase-3 cells-NIM-Cas-3. **B)** ADM217-47 treated placenta showing immunoreactive caspase-3 cells-IM-Cas-3- (X100 magnification).

Involvement of Caspase 3 activation in placental tisuues of $ADM2_{17–47}$ treated pregnant rats:

Activation of caspases is a central mechanism of apoptosis, and caspases are considered to be the ‘‘executioners’’ of cell death. We examined whether the caspase protein is activated in the apoptotic process induced by $ADM2_{17–47}$. Because caspase-3 is a central effector of apoptosis, we examined the proteolytic processing of caspase-3 by western blot analysis. Active caspase-3 fragments were more abundant in $ADM2_{17–47}$ - treated animals compared with untreated controls shown in **Figure7.**

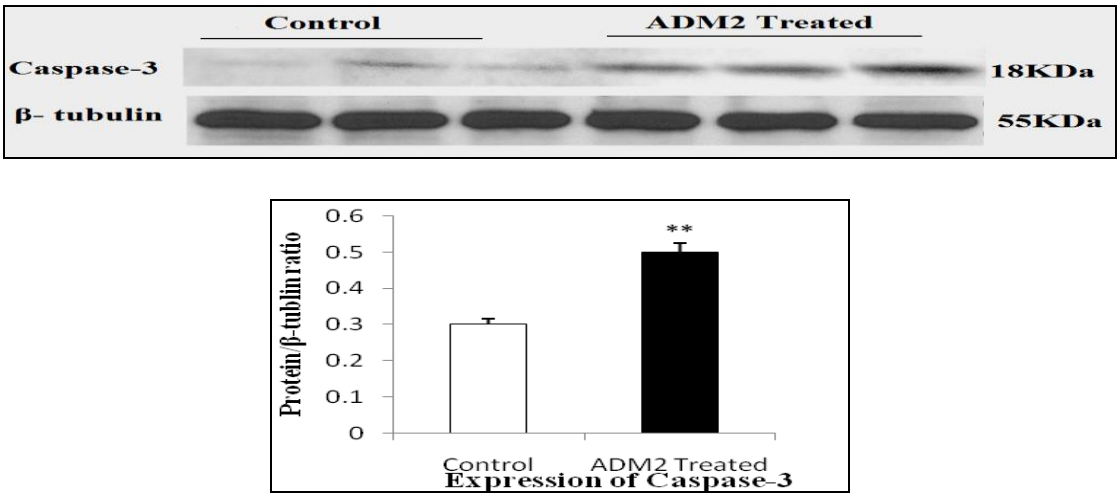

FIG 7: EXPRESSION OF ACTIVE CASPASE-3 IN THE PLACENTA OF ADM217–47-TREATED AND UNTREATED CONTROL RATS.

Top panel: figure: 6A Western blot analysis of cleaved caspase-3 and β-tubulin in placenta of ADM217–47-treated and untreated control rats and in Bottom panel: Densitometry analysis of the respective protein bands normalized to β-tubulin. Data are mean ± SEM values for five to six replicate animals in each group. *$P < 0.05$ indicates significantly different compared with the control

Effect of ADM2 $_{17-47}$ antagonist on CRLR, RAMP1, RAMP2 and RAMP3 mRNA transcripts in placental tissues:

RT-PCR analysis was performed to elucidate the changes in the mRNA expression levels of ADM2 receptors (CRLR, RAMP1, RAMP2 and RAMP3) in the placental tissues of pregnant rats which are treated with ADM2 antagonist at late gestation. The level of mRNA for these component proteins was determined relative to β – actin in placental tissues from each animal. It observed that there is a significant decrease in mRNA expression levels of CRLR and RAMP1, & RAMP3 ($P < 0.05$) but not RAMP2 **(shown in Figure: 8)** compared to controls.

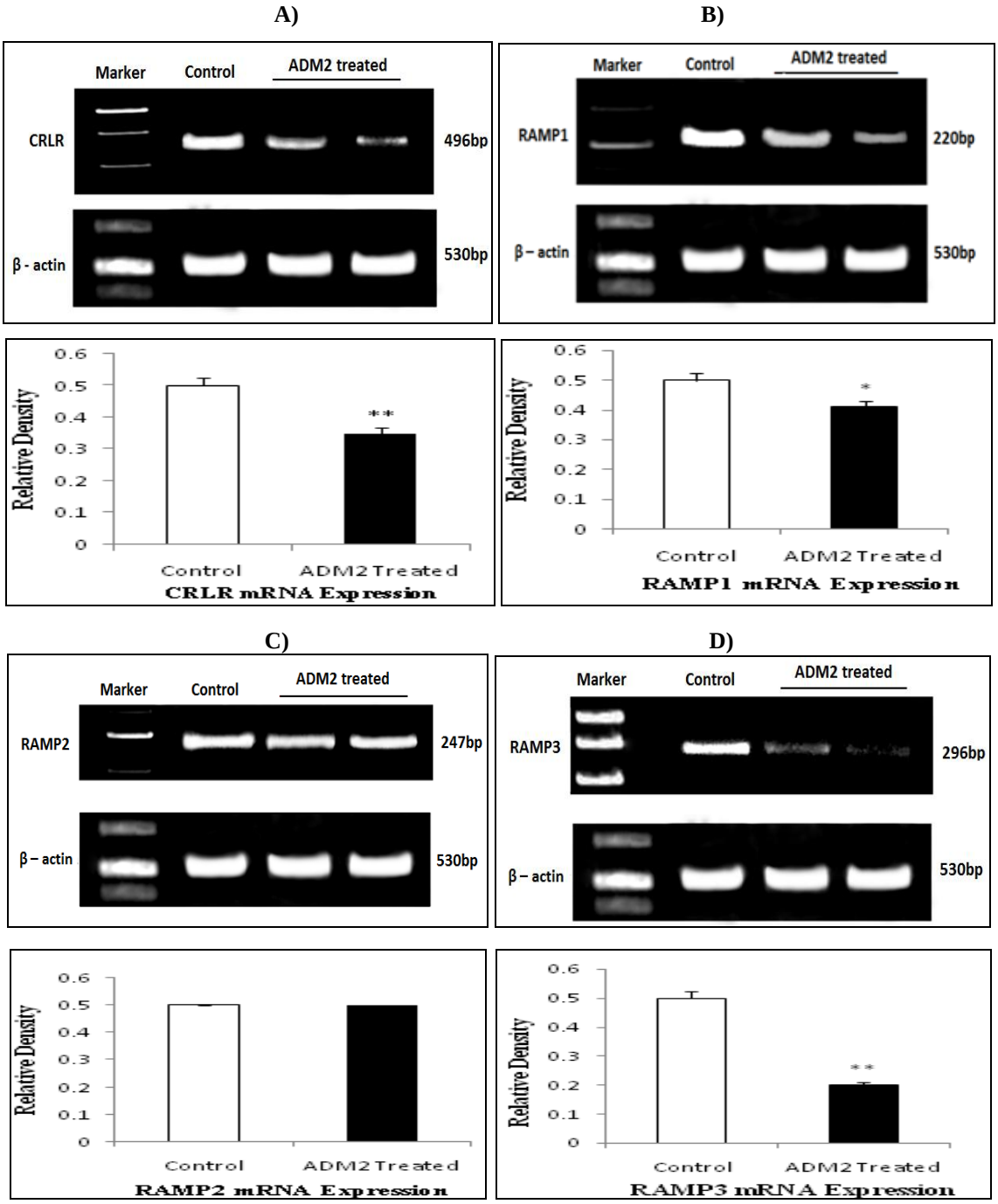

FIG 8: EFFECT OF ADM2 ANTAGONIST ON THE EXPRESSION OF CRLR, RAMP1, RAMP2 & RAMP3 IN THE PLACENTA OF PREGNANT RATS.

Top panel shows mRNA expression of **(A)** CRLR **(B)** RAMP1 **(C)** RAMP2 **(D)** RAMP3 and β- actin analyzed by RT-PCR technique using appropriate primers. Bottom panel shows the densitometry analysis of respective PCR products for CRLR and RAMP1, 2, 3 and the data are presented as a ratio relative to that of β- actin. Bars represent the mean ± SEM from four replicates in each group.*P < 0.05, ** P< 0.001indicates significantly different compared with the control.

DISCUSSION: ADM2/IMD is newly invented novel peptide belongs to CALCA/CALCB peptide family, which is identified by Roh et al. and takei et al.[5] in 2004 using phylogenetic profiling approach [5]. Because of the structure homology and the amino acid sequence of ADM2, it has important regulatory role in homeostasis, vasodilatation and in maintaining normal pregnancy function like ADM & CALCB [23, 24]. It was also reported that these peptides altered m-RNA expressions during pregnancy and causes pathological pregnancies such as hypertension and preeclampsia [25, 26]. Madhu chauhan et al.[15] in 2006 provides the evidence of ADM2 role in fetoplacental development and placental function during pregnancy and in 2011 these authors given another report of ADM2 in early embryonic development in rats [15, 27]. Based on the above authors reports it came to known that ADM2/IMD have emerging roles in reproduction mainly in fetoplacental development in mammals.

In this view the present study was undertaken to demonstrate the role of ADM2 in fetoplacental development in pregnant rats at late gestation period, there was no studies we found on ADM2 and its role during late gestation and our work study differed from those previous study in two aspects. First, the ADM2 antagonist infusion period is differ from previous study, where as in our study infusion started at late gestation period (i.e. day 18 of gestation and ended on day 22 of gestation). Second, we examined steroid hormone levels in the serum and also we observed the m-RNA expression levels of ADM2 receptors.

The major goal of this study was under taken to determine the role of ADM2/IMD during pregnancy especially before the birth of fetus it means at late gestation period in pregnant rats (Third trimester in humans). For this study we infused osmotic mini pumps with ADM2 antagonist at 50 and 200 µg / rat / day on gestational day 18 and were sacrificed on gestational day 22. The main intend of using this infusion process is to block the activity of endogenous ADM2 through continuous exposer of ADM2 antagonist. The present study revealed that there was no fetal resorptions are found both in control and $ADM2_{17-47}$ treated pregnant rats but fetoplacental weights are significantly decreased ($P < 0.05$) in ADM2 antagonist treated rats compared to controls (**Shown in Table .1**) .

The reductions in fetoplacental weights were more substantial with $ADM2_{17-47}$ at 200 µg / rat / day compared with 50 µg / rat / day showing decline compared to the controls. Alternatively, we examined the fetal lengths, total sac weights, uterus weights and amniotic fluid weights. fetal lengths are significantly decreased ($P < 0.05$) follow a trend of decreasing with increasing doses of $ADM2_{17-47}$ showing decline with higher dose compared to controls (**Figure.1**) and total sac weight also decreased compared to controls but no change were found in uterus & in amniotic fluid weights.

Further, we analyzed progesterone and 17β-estradiol in the serum because these two steroid hormones play a key role in fetoplacental development throughout the gestation. Ovaries synthesized progesterone in early gestation to maintains the secretory endometrium later it is necessary for the survival of the embryo development then placenta takes over the production of progesterone during the second and third trimesters of pregnancy for fetal growth and development but sometimes due to pregnancy-related complications or placental disorders there is a decrease in progesterone production presumably by the placenta occurs during the second and third trimester of pregnancy[28] .

In this view we examined progesterone and 17β-estradiol levels in the serum of pregnant rats and it observed that, there was a significant decrease ($P < 0.05$) in serum progesterone and 17β-estradiol levels in ADM2 antagonist treated rats compared to controls (**Figure. 2**).There after we examined two questions: Does infusion of $ADM2_{17-47}$ during late gestation period causes any apoptotic changes and alterations in the m-RNA expression levels of ADM2 receptors in placenta of $ADM2_{17-47}$ treated rats? DNA fragmentation analysis is the primary step to detect the apoptosis in cells because degradation of nuclear DNA (DNA fragments) into nucleosomal units is one of the biochemical hallmarks of apoptotic cell death. So, we examined the possibility of involvement of DNA fragmentation associated with reduction in

placental and fetal growth. Highly fragmented DNA was observed in placental tissues and the degree of percentage of DNA fragmentation was significantly increased ($P < 0.05$) in $ADM2_{17–47}$ treated placentas than in controls (**Figure.3**). Fragmentation in placental tissues were more substantial with $ADM2_{17–47}$ at 200 µg/day compared with 50 µg/day.

Histopathology has been used as an important diagnostic tool in biochemical pathology for many years and has been a corner stone in the large field of biomedical pathology. Histopathological studies are necessary for the evaluation and description of possible damages caused to the rats through infusion of $ADM217_{–47.}$ Hence, infusion of ADM2 antagonist causes severe histopathological changes and was observed in high dose of $ADM2_{17-47}$.

The experimental animals showed impairment of labyrinths & exhibited decreased fetal vascular development and clusters of glycogen cells & spongiotrophoblasts are observed in the basal zone compared to controls **(Figure.4**). karyorrhexis or Pyknosis cells are apoptotic cells which are detectable during early process of apoptosis under light microscope with haematoxylin and eosin stain and chromatin condensation occurs due to pyknosis and it appears when the cell undergoing necrosis or apoptosis [29]. Therefore in our histopathological studies, apoptotic cells characterized by pyknosis or karyorrhexis cells are observed in labyrinth zone and decidus of placenta in $ADM2_{17-47}$ treated rats (**Figure.5**).

Thereafter, activation of caspases conforms the apoptosis because these caspases plays a key role in central mechanism of apoptosis, and caspases are considered to be the ‘‘executioners’’ of cell death. We examined whether the caspase proteins are activated in the $ADM2_{17–47}$ treated placentas. In our immunohistochemical staining studies, we observed the presence of active caspase-3 protein in $ADM2_{17–47}$ treated placental tissues. The active caspase-3 protein seen abundantly in $ADM2_{17–47}$ treated labyrinth and basal zone of placental tissues than in controls (**Figure.6**). Similarly, we examined the proteolytic processing of caspase-3 by western blot analysis. Active caspase-3 fragments were more abundant in $ADM2_{17–47}$ -treated animals when compared with untreated controls (**figure.7**). By this study it came to known that ADM2 antagonist induces apoptosis in placenta and it may leads to fetoplacental growth restriction.

The newly identified AM2 binds non selectively to all three CRLR/RAMP complexes but it has a high potency with CRLR/RAMP1 and CRLR/RAMP3 receptors [5, 11]. It was also reported that ADM2 mRNA expression was observed in trophoblast cells during first-trimester and in human placental villi [14]. Recently Wong et al.[27] demonstrates the expression of intermedin (IMD) and its receptor components in the uterus of the female rat during the estrous cycle and its effect on uterine contraction. Regarding to above references in the present study we probed the mRNA expression levels of ADM2 receptors (CRLR, RAMP1, RAMP2 and RAMP3) in the placental tissues of pregnant rats which are treated with ADM2 antagonist at late gestation.

The level of mRNA for these component proteins was determined relative to β – actin in placental tissues from each animal. The mRNA expression levels were observed in placental tissues, there is a significant decrease in mRNA expression levels of CRLR and RAMP1, & RAMP3 ($P < 0.05$) but not RAMP2 shown in (**Figure.8**) compared to controls.

Placental growth, development, and aging are crucial to the overall well-being of the fetus and are controlled by a range of endocrine signals, peptides growth factors and steroids. Steroid hormones especially progesterone and estrogen maintains the fetoplacental development during pregnancy, it is well known that during third trimester of pregnancy placenta itself produce& regulate the progesterone levels, alternatively estrogen was produced & regulated by progesterone [28].

In this connection the data of present study reveal that infusion of ADM2 antagonist and in down regulated the serum levels of progesterone and 17β-estradiol in pregnant rats during late gestation period due to impact of ADM2 antagonist on placenta in production or regulation of progesterone and 17β-estradiol, which may leads to placental disfunction or placental disorder and may decreases the fetoplacental weights & lengths

&one of the placental dysfunction is apoptosis, or physiological cell death, has been observed in the placenta, particularly in the latter half of pregnancy. Placental apoptosis also increases in association with fetal growth retardation –FGR and placental apoptosis may affect a range of placental functions [28, 29, 30].

In the present study it shown that placental apoptosis was occurred in ADM2 antagonist treated rats over the final third of rat pregnancy, the period of maximal fetal growth. In our DNA fragmentation analysis we showed the evidence of apoptosis occurred in placental tissues and in immunohistopathological studies it was confirmed by pyknosis and karyorrhexis cells in labyrinth zone and deciduas of placenta along with the immunolocalization of caspase3, and increased levels of caspase3 was observed in western blot analysis, it indicates that , apoptotic changes was occurred in ADM2 $_{17\text{-}47}$ treated rats by this analysis it may prove that ADM2 antagonist causes DNA damage in placenta through apoptosis and also alters the steroid hormone levels ,the mRNA expression levels of ADM2 receptors it may leads to fetoplacental growth restriction.

CONCLUSIONS: In conclusion, the present data demonstrate that ADM2 antagonist acts as a non-competitive inhibitor for endogenous ADM2 and inhabited the actions of ADM2 in fetoplacental development and induces apoptosis along with changes in steroid hormone levels & m-RNA expression levels in placenta during rat late gestation period and leads to fetal growth restriction .Therefore our results suggest that ADM2 is required for maintaining normal placental function and fetal growth even at late gestation period.

COMPETING INTERESTS: The authors declare that they have no competing interests

AUTHORS' CONTRIBUTIONS: GLD carried out the all experiments and wrote the manuscript. PJ contributed study conceptualization & participated in the design of the experiments and revising the manuscript. All authors read and approved the final manuscript.

ABBREVIATIONS: ADM2, adrenomedullin 2; IMD, intermedin ; $ADM2_{17\text{-}47}$, adrenomedullin 2 antagonist; CRLR, calcitonin receptor-like receptor; RAMPS, receptor activity modifying proteins; m-RNA; messenger RNA; RT-PCR, reverse transcription PCR; AMV, avian myeloblastosis virus ; cDNA, complementary DNA; Mgcl2,magnesium chloride; bp ,base pairs; DAB, diaminobenzidine, PBS, Phosphate-buffered saline. RIA, Radioimmunoassay.

ACKNOWLEDGEMENTS: Authors are grateful to the Head, Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam, Tirupati for providing lab facilities to carry out research work. Penchalaneni. Josthna acknowledges the University grants Commission (UGC), New Delhi for providing financial assistance (MRP- Ref. No. 37-347/2009 SR). We thank Dr. Venkata Subbaiah Kotakadi, Research Scientist, DST PURSE Centre, Sri Venkateswara University, Tirupati for helpful discussions and technical assistance**.**

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How to cite this article:
Deepika GL and Josthna P: Infusion of Adrenomedullin-2 Antagonist Induces Apoptosis and Changes M-RNA Expression Levels of ADM2 Receptors in Placenta of Pregnant Rats during Late Gestation and Causes Fetoplacental Growth Restriction. Int J Pharm Sci Res 2015; 6(3): 1137-49.doi: 10.13040/IJPSR.0975-8232.6(3).1137-49.