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Bimolecular study to characterize the mRNA for β-adrenoceptor subtypes in BPA

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Abstract:
Background*: β-adrenoceptors have an important physiological function in the regulation of vascular tone and impairment of β-adrenoceptor function is associated with hypertension.*
This study aimed at characterizing of β-adrenoceptor subtypes in in vitro preparations of BPA.
Materials and Methods*: Polymerase chain reaction (PCR) studies used to determine the mRNA expression for β-adrenoceptors subtypes using bovine-specific primer pairs.*
Results*: The total RNA concentrations in triplicate BPA samples were (28.89ng/µL, 31.67 ng/µL and 35.45ng/µL). The A260/A280 ratios were 2.3, 2.72 and 2.47 respectively. Agarose gel electrophoresis showed PCR products with two bands at the expected sizes corresponding to β1- & β2-adrenoceptors, β1-adrenoceptor (160 bp) and β2-adrenoceptor (202 bp), with no bands corresponding to the β3-adrenoceptor in BPA samples.*
Conclusion: *This research, using functional studies supported with PCR investigations, indicated that the relaxation of BPA was mediated via classical β1/β2-adrenoceptors, which seem to be presented throughout vasculature smooth muscle layer, with proof opposing the involvement of atypical β-adrenoceptors (β3 and atypical β1-adrenoceptors state).*
Key Word*: β-adrenoceptors; Polymerase chain reaction (PCR); primer.*

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I. Introduction

β-adrenoceptors have an important physiological function in the regulation of vascular tone as they mediate the vasorelaxation response to endogenous catecholamines. Impairment of β-adrenoceptor function is associated with hypertension in animal models and in humans[1] and dilated cardiomyopathy and may be associated with the autoimmune disease and the presence of anti-β-adrenoceptor antibodies[2,3].

Although vascular β-adrenoceptors were initially categorized as β2-adrenoceptors[4] and β2-adrenoceptors appear to predominate in most vessels. β1-adrenoceptors have also been reported to play a role in vasodilation[5,6] and have been reported as the predominant subtype in some vessels such as, cerebral arteries[7], coronary arteries[8], murine femoral and pulmonary arteries[9] and smooth muscle cells of mesenteric arteries[10]. Additionally, Wellstein et al., (1988) has reported that β1-adrenoceptors may have a dominant effect over that of β2-adrenoceptors[11].

Lands et al., (1967) originally classified β-adrenoceptors into β1/ β2-adrenoceptors4 and subsequently Harms et al., (1977) proposed the presence of ‘’atypical’’ β-adrenoceptors in rat adipocytes[12].

Atypical β-adrenoceptors were eventually defined as β3-adrenoceptors as well as putative β4-adrenoceptors that were subsequently recognised as a low affinity state of the β1-adrenoceptor[13] with a clear role in the gastrointestinal tract and adipose tissue.

Although there have been many studies to characterize vascular β-adrenoceptor subtypes in different blood vessels from different animals, only a few studies have been conducted on bovine blood vessels with none aimed at classifying β-adrenoceptors in bovine pulmonary arteries (BPA). Previous studies have indicated the presence of β-adrenoceptors in bovine long posterior ciliary artery[14], β1 and β2- adrenoceptors as well as atypical β-adrenoceptors in cultured endothelial cells from BPA[15] in both cultured smooth muscle and endothelial cells from bovine aorta[17] and in bovine aortic smooth muscle[16].

To evaluate the β-adrenoceptor subtypes in BPA polymerase chain reaction (PCR) studies used to determine the mRNA expression for β-adrenoceptors subtypes.

II. Material And Methods

Polymerase chain reaction (PCR) studies used to determine the mRNA expression for β-adrenoceptors subtypes using bovine-specific primer pairs.

Physiological salt solution (PSS): All experiments used modified Krebs-Henseleit Physiological salt solution (PSS), of the following composition (milimolar, mM): NaCl (119), KCl (4.7), NaHCO3 (24.8), MgSO4 (1.2), KH2PO4 (1.2), CaCl2 (2.5) and glucose (11.1), which was prepared daily.

Tissues harvesting, Collection and preparation: Bovine lungs from different animals under 20 months were dissected within 50 minutes of slaughter. Dissection was carried out to obtain arterial segments from the third and fourth generation of conventional arteries that were immediately stored on ice-cold PSS during the dissection procedure and then transferred to RNAlater®, an RNA stabilizing solution that prevents RNAases degrading the RNA, for storage until required. Samples can be kept in RNAlater® solution for one week at 25ºC, up to a month at 4ºC and for long term at -20ºC or -80º. Tissues were divided in three groups to make three samples.

Bimolecular studies: To avoid ribonucleic acid (RNA) contamination/degradation the bench was cleaned with ethanol before starting any work and gloves were worn while handling tissue and during RNA extraction.

1. The total RNA extractions: Total RNA was isolated from BPA in accordance with the instructions provided with the High Pure RNA Tissue kit (RocheDiagnostics, Mannheim, Germany). Briefly, arteries were disrupted in a lysis/binding buffer using a glass-glass homogenizer. Tissue was further disrupted by passing the homogenate slowly (5 – 10 times) through a 21-gauge sterile syringe needle inserted into a plastic syringe. The Lysis/Binding buffer also contains guanidine hydrochloride, which is required for denaturing the tissue as well as inactivating any RNAase activity to maintain intact RNA in the sample. The homogenate was centrifuged to separated proteins and cell debris. The remaining supernatant was treated with ethanol to promote flocculation of the RNA.

RNA was separated from the lysate supernatant by adding it to a "High Pure Filter tube" in presence of guanidine HCl and centrifuged. The glass fiber filter has a high affinity for RNA/DNA. The retained DNA (deoxyribonucleic acid) was subsequently digested by the addition of DNaseI for 15 min at room temperature. The DNase was then deactivated by the addition of a deactivating buffer and then removed from the filter by centrifugation followed by several washes. Finally, pure RNA was eluted from the filter with the elution buffer, collected and maintained on ice for further quantification or alternatively was stored at -80ºC (Figure 1).

The samples of BPA extracted RNA were assessed using a spectrometer (Gen5 Take3) with absorbance measured at 260nm and 280nm. The ratio A260/A280 was taken as RNA purity estimation and RNA concentration in ng/µl.

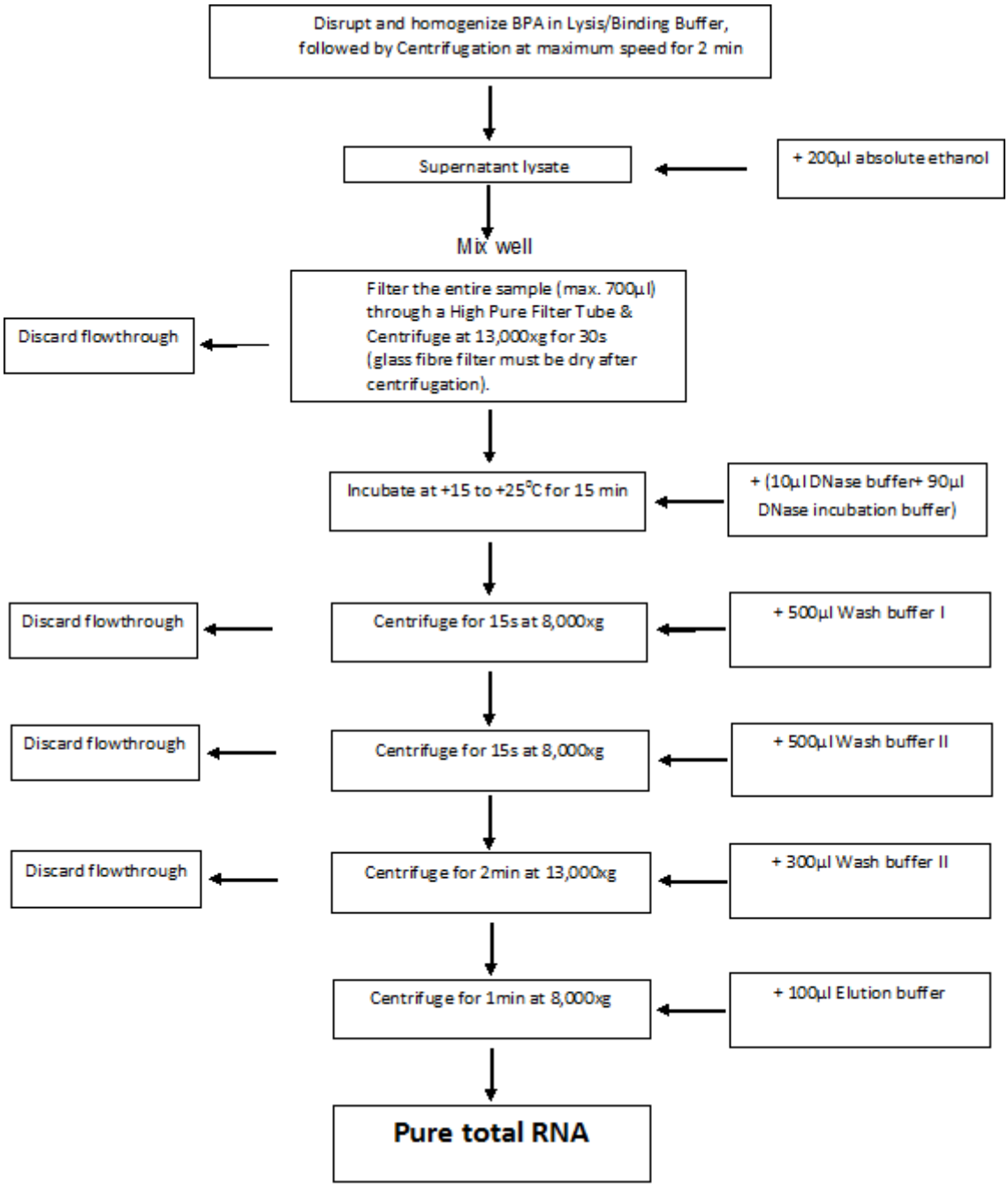

Figure 1: Total RNA extraction according to High Pure RNA Tissue kit. (RocheDiagnostics, Mannheim, Germany).

2. Retro-transcription: Retro-transcription is the process of producing complementary DNA (cDNA) from isolated messenger RNA (mRNA). In order to get an optimal cDNA yield, the total RNA was kept pure and free from any genomic DNA or RNase by ensuring that all working surfaces were cleaned with ethanol and by using RNase-free ware and reagents and by wearing gloves to avoid contamination.

Retro-transcription was carried out according to the instructions that accompanied the Transcriptor High Fidelity cDNA Synthesis Kit (RocheDiagnostics, Mannheim, Germany) and outlined in figure 2.2. Briefly, all reactions were carried out in nuclease-free thin walled 0.2 ml PCR tubes. The template-Primer mixture was prepared by adding Anchored-oligo (dt)18 Primer or Random Hexamer Primer to the total RNA and adjusting the final volume with PCR-grade water. The secondary structure of the RNA was denatured to produce single-stranded RNA using the thermocycler (cycle: 650C for 10 min, 40C cooling to keep the sample till the collection time). Thereafter, the sample was kept on ice while the remaining reagents (Reverse Transcriptase Reaction Buffer, Protector RNase Inhibitor, Deoxynucleotide Mixture, DDT and Reverse Transcriptase) were added. This was followed by another incubation in thermo-cycler for 2 steps:

Step 1: 550C, 30 min.
Step 2: 850C for 10 min to inactivate the Reverse Transcriptase. Finally, the reaction mixture was transferred to ice to cool and then stored at -200C for later PCR analysis.(Figure 2).

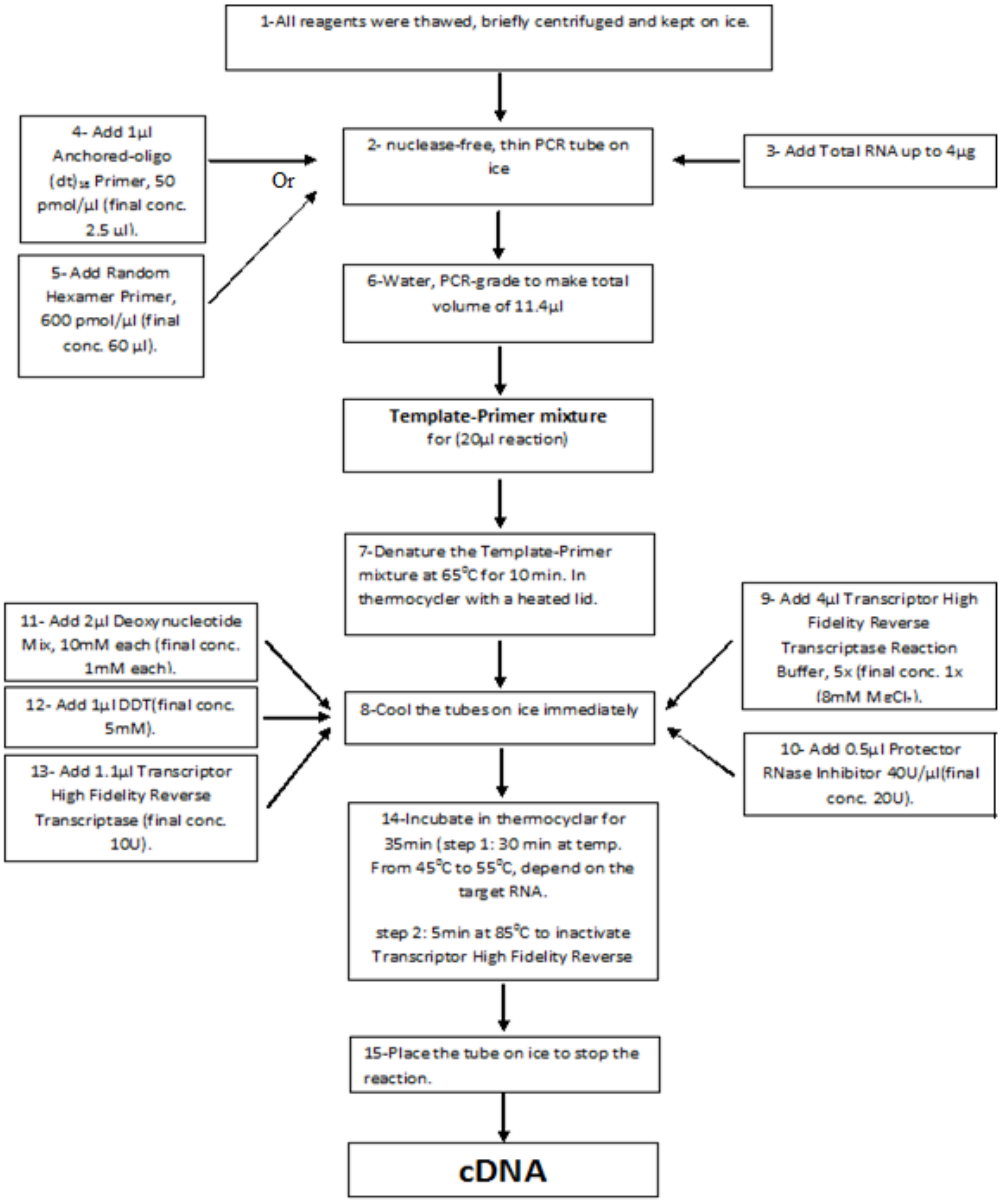

Figure 2: cDNARetro-transcription according to Transcriptor High Fidelity cDNA Synthesis kit. (RocheDiagnostics, Mannheim, Germany).

3. Standard Polymerase chain reaction (PCR): Polymerase chain reaction (PCR) is a process that allows amplification of DNA. In the present study the technique was used to amplify the gene(s) that underlie the expression of particular receptors from the cDNA[17]. Briefly, the double-stranded cDNA is denatured into single stranded templates by exposure to high temperature (95oC). The temperature is then reduced to certain annealing temperature (see related chapters for the annealing temperature for each primer) which allows the oligonucleotide primers to anneal to the desired regions of the DNA and this facilitates the binding of a thermo-stable polymerase enzyme at the region of interest. The temperature is then adjusted to 72oC, which is the optimal temperature for the polymerase activity and DNA elongation resulting in doubling the double stranded DNA and gene(s) of interest. The cycle of temperature change is repeated continuously to amplify the DNA. Because the generation of double-stranded DNA is exponential; large copies of DNA (µg) are produced rapidly from nanograms (ng) amount of the specific DNA template sequence (Figure 3).

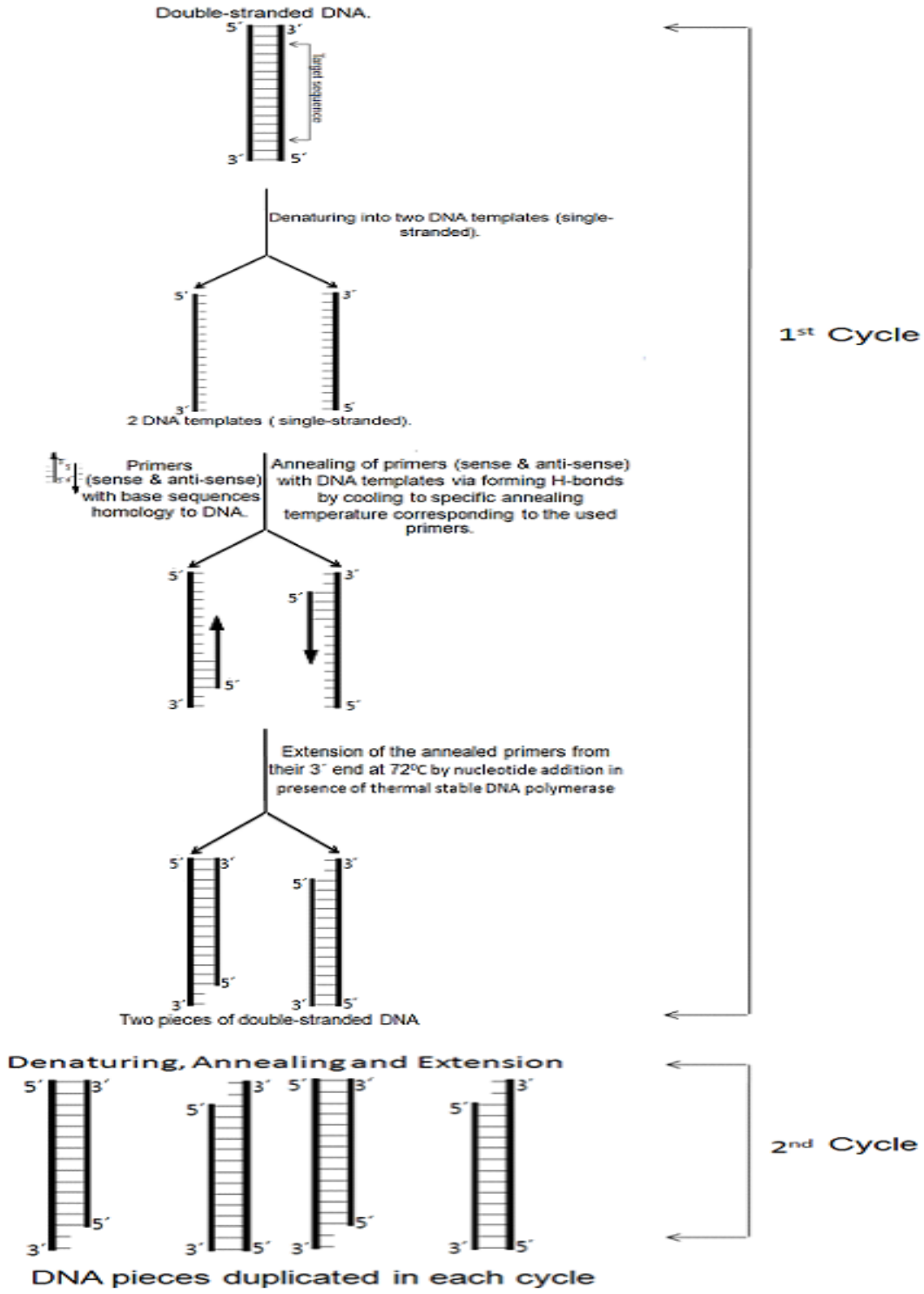

Figure 3: Polymerase chain reaction steps

Oligonucleotide primers

Oligonucleotide primers are specific small DNA fragments that have a base sequence that is homologous with the termination of the DNA of interest. Different primer pairs (sense and antisense primer) for the for β1, β2 and β3-adrenoceptors, were used to trigger the amplification of only the cDNA and not the genomic DNA (gDNA) as the sense and antisense primers for each target gene were placed in two different exons of the gene. So the length of the PCR products of cDNA and gDNA differed.PCR was conducted with bovine-specific primer pairs (sense and antisense primer were purchased from Eurogentec) for β1, β2 and β3-adrenoceptors, listed in table (1)[18] for cDNA selective amplification.

Table 1: Sequences of PCR primer pairs (f = forward; r = reverse), Primer length, product size, CDS (coding sequences) and the annealing temperatures

Sequence (5'→3')	Primer length (base)	product size (bp)	CDS	Annealing temperature
β_1 f TCGCCCTTCCGCTACCAGA	19	160	566-584	62.0^0C
β_1 r ACTCGGGGTCGTTGTAGCA	19		707-725	60.0^0C
B_2 f TCATGTCGCTTATTGTCCTGG	21	202	116-136	47.3^0C
B_2 r CACCAGAAGTTGCCAAAAGTCC	22		296-317	49.7^0C
B_3 f ACCGTGGGAGGCAACCTG	18	155	151-168	60.0^0C
B_3 r TGGCCGGTCAGCGCCAA	17		289-305	58.0^0C

Protocol for PCR: The PCR process was carried out as outlined in figure 4. A negative control reaction was prepared in parallel by swapping the cDNA for PCR-grade water.

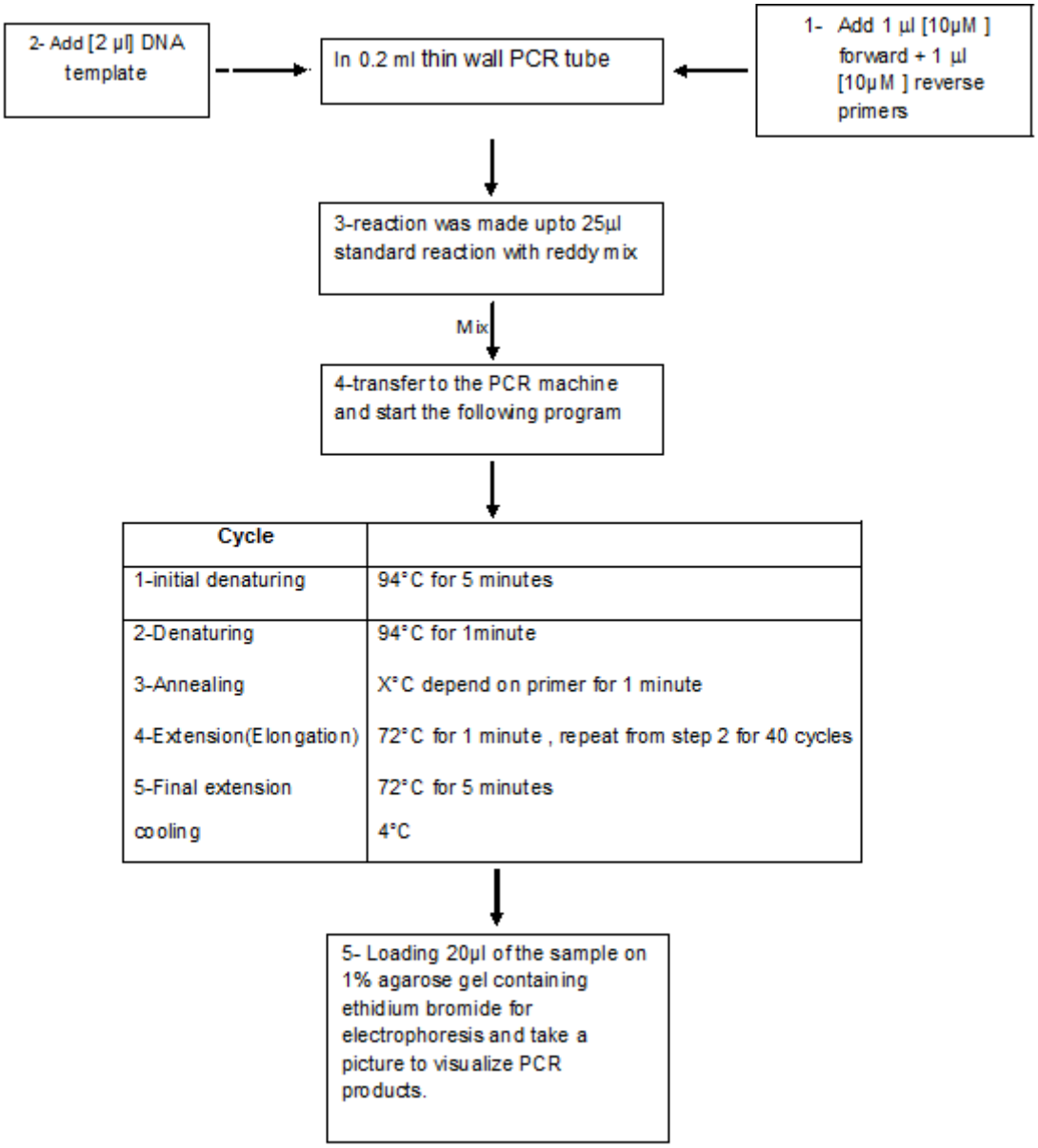

Figure 4: Standard Polymerase Chain Reaction protocol.

Gel Electrophoresis: PCR products were loaded into the wells of a 1% agarose gel matrix, prepared as outlined in figure 5. In this technique negatively charged DNA is separated according to size. A voltage (100 Volt, V) is applied across the longitudinal axis of the gel, which attracts the DNA to the anode. Smaller fragments of DNA are able to sieve through the agarose gel matrix more easily, and therefore faster, than larger fragments. A

commercially available DNA size ladder was included in each gel to allow comparison of the separated DNA. The DNA was visualized by exposing the gel to ethidium bromide, which binds to DNA. Under UV light, using a trans-illuminator, the ethidium bromide fluoresces allowing a visual image of the DNA, which was recorded by digital camera.

Gel electrophoresis was conducted against 100bp DNA ladder (purchased from life technologies, Invitrogen company) on 1% agarose gel (90 min, 100v). PCR products were visualized using ethidium bromide. Negative control (cDNA free) was also incorporated to test for contamination.

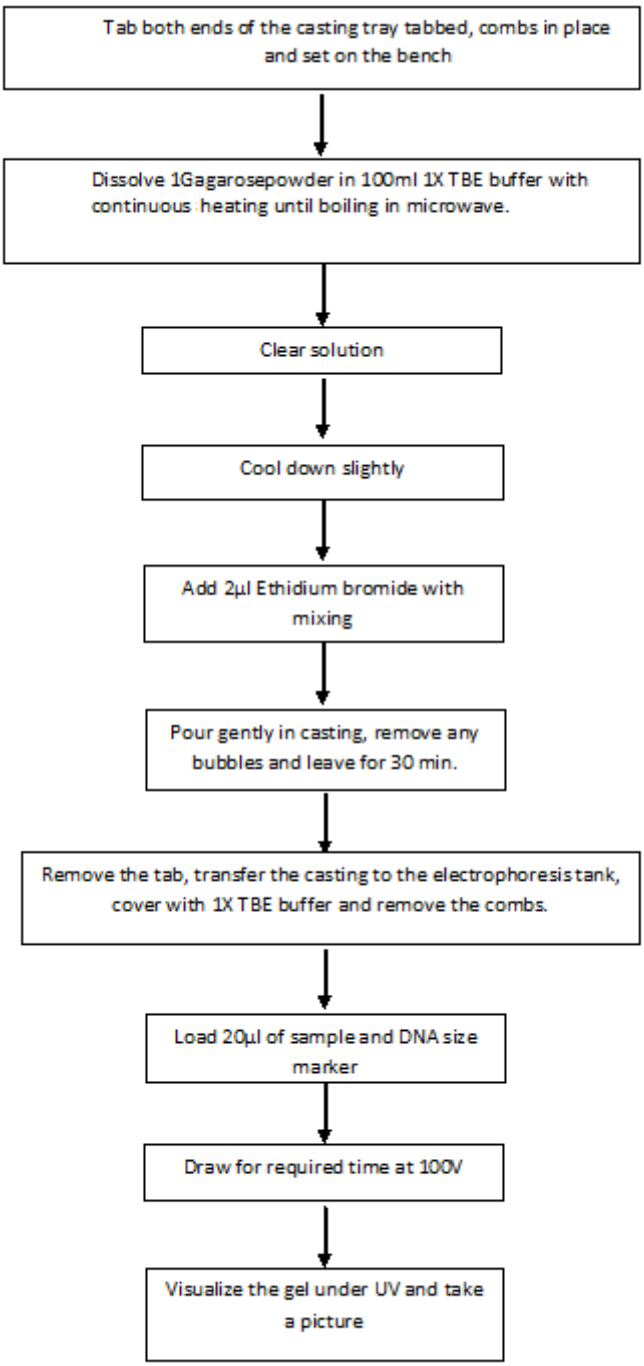

Figure 5: The Gel Electrophoresis of PCR products.

Data analysis:

1. **Total RNA Quantification:** A 1microliter (µl) sample of extracted RNA was used to assess concentration and purity, which was measured using a micro spectrometer (BioTek, Gen5 Take3). RNA concentration was assessed by measuring the absorbance (A) of transmitted ultraviolet (UV) light at 260 nanometre (nm). RNA absorbs UV light therefore the absorbance is directly proportional to the concentration of RNA (ng/µL).The purity of the sample is determined by measuring the ratio between the absorbance at 260nm and 280nm. Protein impurity in the sample absorbs light at 280nm. The ratio A260/A280 reflects [RNA]/ [protein] and a value of around 2 was considered a pure RNA sample. The instrument (BioTek, Gen5 Take3) was calibrated with 1µl RNase-free water before measuring the sample.
2. **Size of PCR products:** The expected size of each PCR product in base pair, was calculated from corresponding gene sequence and oligonucleotide primer, since it equal the number of bases pairs in the coding sequence of the published gene sequence, starting from forward primer annealing site to that of reverse. PCR product size estimation is important in selection of the DNA size marker as well as expected gel running time and may indicating adjust the voltage as well. The actual size of separated PCR products, which should equal to the expected size, was calculated by comparison with the adjacent DNA size marker band in base pair (bp). Whereas the negative control should not show any band as it should not get any amplification products, otherwise it indicates contamination.

III. Result

Total RNA quantification and qualification: The total RNA concentrations in triplicate BPA samples were (28.89ng/µL, 31.67 ng/µL and 35.45ng/µL). The A260/A280 ratios were 2.3, 2.72 and 2.47 respectively.

Agarose gel electrophoresis showed PCR products with two bands at the expected sizes corresponding to β1- & β2-adrenoceptors, β1-adrenoceptor (160 bp) (Figure 5) and β2-adrenoceptor (202 bp) (Figure 6), with no bands corresponding to the β3-adrenoceptor in BPA samples (Figure 7). Moreover no bands were associated with the negative controls for all primers, thus ensuring that there was not contamination with genomic DNA and the detected products, and the bands at the expected sizes corresponding to β1- & β2-adrenoceptors, were from BPA mRNA.

Although the PCR product with a bands of size relating to β2-adrenoceptor (202 bp) in Figure 7 can be used as a positive control for the PCR running to ensure that there was not issue with gel running. There was not positive control for β3-adrenoceptor primer. Therefore the absence of β3-adrenoceptor related band could be either because of absence of β3-adrenoceptor mRNA in the samples or because of an issue in the related primer.

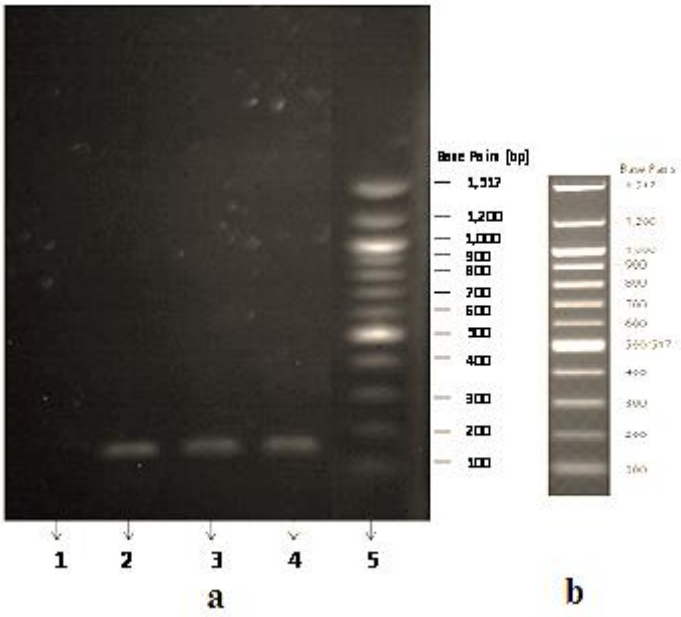

Figure 5: (a) Ethidium bromide-stained agarose gel where (1) = negative control, (2, 3, 4) =PCR products (160bp) amplified from cDNA reverse-transcripted from three mRNA extractions using β1-adrenoceptor specific oligonucleotide and (5) =the ladder of size marker in bp. (b) hyperladder reference (Invitrogen company).

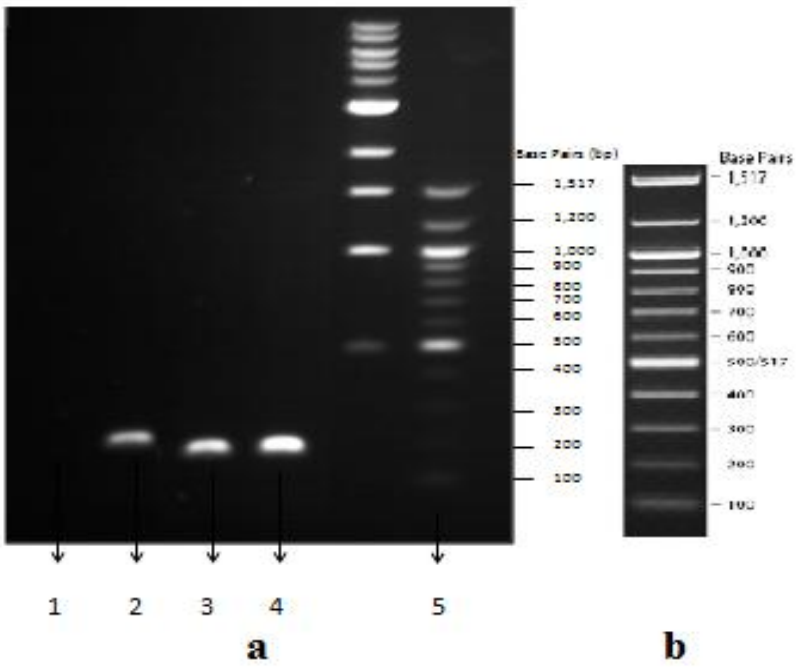

Figure 6: (a) Ethidium bromide-stained agarose gel where (1) = negative control, (2, 3, 4) =PCR products (202bp) amplified from cDNA reverse-transcripted from three mRNA extractions using β2-adrenoceptor specific oligonucleotide and (5) =the 100bp hyperladder. (b) hyperladder reference (Invitrogen company).

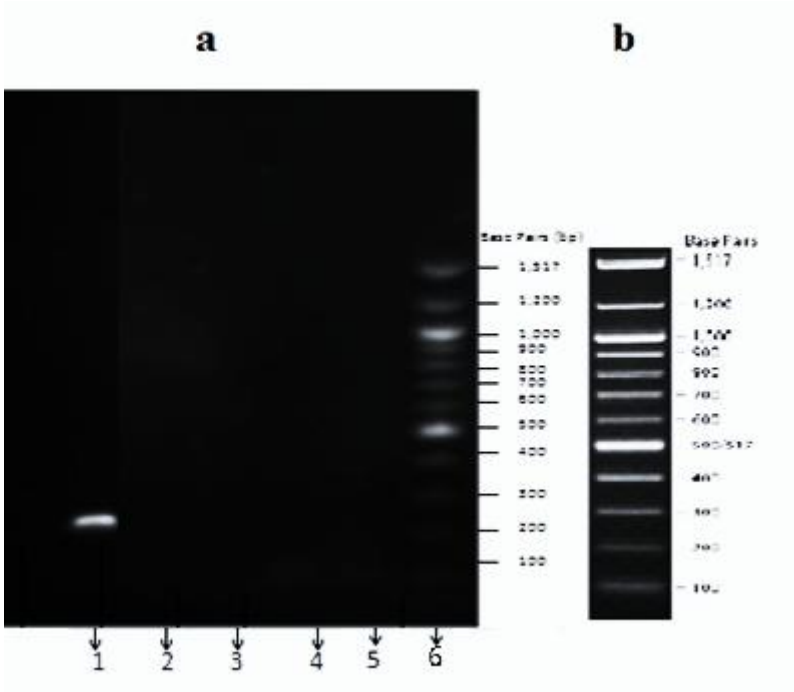

Figure 7: (a) Ethidium bromide-stained agarose gel where (1) = positive control for β2-adrenoceptor expected PCR product (202bp), (2) = negative control, (3, 4, 5) =PCR negative products expected size was (155bp) and the cDNA reverse-transcripted from three mRNA extractions using β3-adrenoceptor specific oligonucleotide and (6) =the 100bp hyperladder. (b) hyperladder reference (Invitrogen company).

IV. Discussion

McKenzie et al. (2010) have reported that in BPA isoprenaline induced a concentration-dependent full relaxation of BPA pre-constricted with U46619[19]. This research, using PCR investigations, indicated that the relaxation may be mediated via classical β1/β2-adrenoceptors, which seem to be presented throughout vasculature smooth muscle layer, with pharmacological proof opposing the involvement of β3-adrenoceptors.

This is in agreement with what has been reported by Ahmed et al. (1990), who proved the presence of β1/β2-adrenoceptors in cultured endothelial cells from bovine pulmonary artery15. The current study also suggests that the β2-adrenoceptor appears to play role in isoprenaline-induce relaxation of BPA, which is in agreement with the findings of Dickens & Morris (1998) using smooth muscle cells cultured from BPA[20] and is also consistent with general view that in vasculature that the majority of β- adrenoceptors is β2- adrenoceptor[4, 21].

Although the absence of mRNA for the β3- adrenoceptor in the PCR studies, could not rely on the PCR results alone because of lacking to the positive control in this study.

Endothelial β-adrenoceptor have also been reported to be present in other bovine blood vessels for example Dümmler et al. (1995) reported the presence of β- adrenoceptors in smooth muscle and endothelial cell cultures derived from bovine aorta16 and Leblais et al. (2008) reported the presence of β2-adrenoceptors in the endothelium[22], whereas β1- adrenoceptors were expressed in the smooth muscle in mice pulmonary arteries. This view supports the observation by Dickens et al. (1998) who reported the presence of β1& β2-adrenoceptors in a smooth muscle cell culture derived from BPA[20].

All together are indicating that the vasodilation responses to isoprenaline in BPA mediated via classical β-adrenoceptors without involvement of atypical β-adrenoceptors.

V. Conclusion

This research, using PCR investigations, indicated that the relaxation of BPA was mediated via classical β1/β2-adrenoceptors, with pharmacological proof opposing the involvement of atypical β3 adrenoceptors.

References

[1]. Matsumoto, T., Szasz, T., Tostes, R.C. & Webb, R.C. 2012, "Impaired beta-adrenoceptor-induced relaxation in small mesenteric arteries from DOCA-salt hypertensive rats is due to reduced KCa channel activity", Pharmacological Research : The Official Journal of the Italian Pharmacological Society, Vol. 65, no. 5, pp. 537-545.

[2]. Abdelkrim, M.A., Leonetti, D., Montaudon, E., Chatagnon, G., Gogny, M., Desfontis, J.C., et al. 2014, "Antibodies against the second extracellular loop of beta(1)-adrenergic receptors induce endothelial dysfunction in conductance and resistance arteries of the wistar rat", International Immunopharmacology, Vol. 19, no. 2, pp. 308-316.

[3]. Patel, P.A. & Hernandez, A.F. 2013, "Targeting anti-beta-1-adrenergic receptor antibodies for dilated cardiomyopathy", European Journal of Heart Failure, Vol. 15, no. 7, pp. 724-729.

[4]. Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P. & Brown, T.G.,Jr 1967, "Differentiation of receptor systems activated by sympathomimetic amines", Nature,Vol. 214, no. 5088, pp. 597-598.

[5]. Nakane, T., Tsujimoto, G., Hashimoto, K. & Chiba, S. 1988, "Beta adrenoceptors in the canine large coronary arteries: Beta-1 adrenoceptors predominate in vasodilation", The Journal of Pharmacology and Experimental Therapeutics, Vol. 245, no. 3, pp. 936-943.

[6]. Zwaveling, J., Winkler Prins, E.A., Pfaffendorf, M. & van Zwieten, P.A. 1996, "The influence of hyperthyroidism on beta-adrenoceptor-mediated relaxation of isolated small mesenteric arteries", Naunyn-Schmiedeberg's Archives of Pharmacology, Vol. 353, no. 4, pp. 438-444.

[7]. Edvinsson, L. &Owman, C. 1974, "Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries in vitro", Circulation Research, Vol. 35, no. 6, pp. 835-849.

[8]. Macdonald, P.S., Dubbin, P.N. & Dusting, G.J. 1987, "Beta-adrenoceptors on endothelial cells do not influence release of relaxing factor in dog coronary arteries", Clinical and Experimental Pharmacology & Physiology, Vol. 14, no. 6, pp. 525-534.

[9]. Rohrer, D.K., Chruscinski, A., Schauble, E.H., Bernstein, D. &Kobilka, B.K. 1999, "Cardiovascular and metabolic alterations in mice lacking both beta1- and beta2-adrenergic receptors", The Journal of Biological Chemistry, Vol. 274, no. 24, pp. 16701-16708.

[10]. Briones, A.M., Daly, C.J., Jimenez-Altayo, F., Martinez-Revelles, S., Gonzalez, J.M., McGrath, J.C., et al. 2005, "Direct demonstration of beta1- and evidence against beta2- and beta3-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries", British Journal of Pharmacology, Vol. 146, no. 5, pp. 679-691.

[11]. Wellstein, A., Belz, G.G. & Palm, D. 1988, "Beta adrenoceptor subtype binding activity in plasma and beta blockade by propranolol and beta-1 selective bisoprolol in humans. evaluation with schild-plots", The Journal of Pharmacology and Experimental Therapeutics, Vol. 246, no. 1, pp. 328-337.

[12]. Harms, H.H., Zaagsma, J. & de Vente, J. 1977, "Differentiation of beta-adrenoceptors in right atrium, diaphragm and adipose tissue of the rat, using stereoisomers of propranolol, alprenolol, nifenalol and practolol", Life Sciences, Vol. 21, no. 1, pp. 123-128.

[13]. Kozlowska, H., Szymska, U., Schlicker, E. &Malinowska, B. 2003, "Atypical beta-adrenoceptors, different from beta 3-adrenoceptors and probably from the low-affinity state of beta 1-adrenoceptors, relax the rat isolated mesenteric artery", British Journal of Pharmacology, Vol. 140, no. 1, pp. 3-12.

[14]. Nyborg, N.C. & Nielsen, P.J. 1995, "Beta-adrenergic receptors regulating vascular smooth muscle tone are only localized to the intraocular segment of the long posterior ciliary artery in bovine eye", Survey of Ophthalmology, Vol. 39 Suppl 1, , pp. S66-75.

[15]. Ahmad, S., Chretien, P., Daniel, E.E. & Shen, S.H. 1990, "Characterization of beta adrenoceptors on cultured endothelial cells by radioligand binding", Life Sciences, Vol. 47, no. 25, pp. 2365-2370.

[16]. Graf, K., Dummler, U., Grfe, M., O'Connor, A., Fleck, E. & Kunkel, G. 1995, "Effect of theophylline on beta-adrenergic receptor density and cAMP content in bovine aortic smooth muscle cells", Inflammation Research : Official Journal of the European Histamine Research Society, Vol. 44, no. 8, pp. 323-326.
[17]. Higuchi, R., Krummel, B. & Saiki, R.K. 1988, "A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions", *Nucleic Acids Research*, Vol. 16, no. 15, pp. 7351-7367.
[18]. Inderwies, T., Pfaffl, M.W., Meyer, H.H., Blum, J.W. &Bruckmaier, R.M. 2003, "Detection and quantification of mRNA expression of alpha- and beta-adrenergic receptor subtypes in the mammary gland of dairy cows", Domestic Animal Endocrinology, Vol. 24, no. 2, pp. 123-135.
[19]. McKenzie, C., Alapati, V.R., MacDonald, A. & Shaw, A.M. 2010, "Mechanisms involved in the regulation of bovine pulmonary vascular tone by the 5-HT1B receptor", British Journal of Pharmacology, Vol. 159, no. 1, pp. 188-200.
[20]. Dickens, G.R. & Morris, P.E. 1998, "Cyclosporine-induced beta-adrenergic receptor down-regulation in bovine pulmonary artery smooth muscle cells: A pilot study", Pharmacotherapy, Vol. 18, no. 2, pp. 341-344.
[21]. Brawley, L., Shaw, A.M. & MacDonald, A. 2000, "Beta 1-, beta 2- and atypical beta-adrenoceptor-mediated relaxation in rat isolated aorta", British Journal of Pharmacology, Vol. 129, no. 4, pp. 637-644.
[22]. Leblais, V., Delannoy, E., Fresquet, F., Begueret, H., Bellance, N., Banquet, S., et al. 2008, "Beta-adrenergic relaxation in pulmonary arteries: Preservation of the endothelial nitric oxide-dependent Beta2 component in pulmonary hypertension", Cardiovascular Research, Vol. 77, no. 1, pp. 202-210.

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