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# Hypertension study in anesthetized rabbits: Protocol proposal for $AT_1$ antagonists screening

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Running title: Protocol proposal for $AT_1$ antagonists screening

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***Abstract***

Introduction: Aim of this study was to establish an optimized fast and safe protocol for the pharmacology screening of $AT_1$ antagonists.

Materials and Methods: The pharmaceutical prototype $AT_1$ antagonist losartan, its active metabolite EXP 3174 and the synthetic compound MM1 were analyzed in order to validate the protocol. Ang II was continuously infused while the animals received the drugs in two procedures.

Results: In the *post*-treatment procedure drugs were administered either in a single bolus dose or in a sequential manner. When losartan was administered in a single bolus dose, efficiency was evident until the $7^{th}$ min (p=0.012) whilst EXP3174 infusion extended the efficiency up to the end of the study (p=0.006). Additionally the sequential injections of losartan prolonged the inhibitory time interval until the end of the study (p=0.045). In the *pre*-treatment procedure animals were treated with two different doses before the infusion of Ang II and results suggested a dose dependent inhibitory effect for both antagonists.

Conclusions: The proposed protocol appears to be safe, simple and fast for the pharmacology screening of $AT_1$ antagonists and enables the evaluation of new antagonists using lower doses than any other one reported in the literature.

*Key-words:* losartan, EXP 3174, $AT_1$ antagonist, hypertension, animal model

***Introduction***

The renin-angiotensin-aldosterone system (RAAS) plays an important role in the regulation of blood pressure. Recognized actions of this hormonal system contributing to blood pressure increase include peripheral vasoconstriction, sodium retention, and stimulation of fluid intake. [1,2] The peptide angiotensin II (Ang II) formed within RAAS, is a vasoconstrictive hormone which leads to blood pressure increase even if it is exogenous administered. [3]

Previous studies so far have shown that blood pressure elevation after exogenous Ang II administration is dose–depended after either a single bolus or a continuous infusion. [4,5] However, termination of the Ang II infusion results in a return of mean arterial pressure to the normal levels within 10-20 seconds, an effect that is consistent with its half-life. [6]

Design of non-peptide $AT_1$ antagonists has been pursued for the past 20 years. The first available $AT_1$ antagonist approved in 1995 was losartan, an orally active antagonist with a selective and competitive profile. [7-9] The effectiveness of losartan has been studied in various animal models using different protocols. [10,11] Losartan inhibits the release of aldosterone [12] and abolishes the vasoconstriction that Ang II causes; thus, it results in blood pressure decrease. [13-15] When absorbed, losartan is metabolized in the liver to the active metabolite EXP3174. The metabolism of losartan has been studied both in humans and animals subjects. [16,17]

A number of $AT_1$ antagonists derived from losartan, such as irbesartan, telmisartan, candesartan, valsartan and olmesartan have been approved for the treatment of arterial hypertension during the last seven years. After the development of a novel receptor antagonist, identification of the *in vitro* affinity of the antagonist to its receptor is mandatory. Unfortunately, the *in vitro* results are not always correlated with the *in vivo* pharmacologic potency of a tested receptor antagonist. Although a high affinity of a drug to its receptor is very important, it is not the only requirement needed for a production of a potent antagonist *in vivo*. The discrepancies between *in vitro* and *in vivo* studies can be attributed to pharmacokinetics and pharmacodynamics of novel compounds.

Animal models have been widely used in order to study the pathogenesis of hypertension and the discovery of new therapeutic procedures. [18,19] An ideal animal model should share similar

cardiovascular anatomy and physiology with humans, resulting in hypertension development. Several protocols as well as different species such as rats, rabbits and dogs have been extensively used in the past for the determination of blood pressure lowering activity. [20-23] Although, Ang II has been infused both in conscious and anaesthetized animals in numerous studies, [24,25] anaesthetized rabbits were used only in a few hypertensive studies while the antihypertensive effect of drugs that precede or follow the Ang II infusion have been hardly documented. [26,27]

It is well known that a fast and safe protocol for the initial screening of the developed antihypertensive compounds is significant. Aim of this research activity is to establish a quick optimized protocol for the pharmacology screening of $AT_1$ antagonists. We tested a quick, un-stressful and painless protocol in an acute experimental setting, using low doses of the compounds under investigation as their availability is limited in the early drug development. In our study, continuous infusion of Ang II in anaesthetized rabbits resulted in hypertension. In order to demonstrate the appropriateness and safety issues of the proposed protocol, we initially validated our protocol regarding both the effects of continuous Ang II infusion and the efficacy of the tested molecules compared with control. Finally, we tested two modes of compound administration, one post- and one pre-treatment with Ang II, in order to acquire an ideal and safe screening of $AT_1$ antagonists.

***Methods***

Seven groups (0, I, $II_A$, $II_B$, $III_A$, $III_B$ and IV) of normotensive adult male, New Zealand white rabbits were used weighing from 2.5 to 3.3 kg each. Groups 0 and I served as the vehicle groups, while Groups II and III served as the main experiment groups. Four to five rabbits were used for each experiment. All procedures were approved by the Institutional Committee Governing Animal Care. Each rabbit was anaesthetized by sodium thiopentone injection (30 mg/kg, Pentothal, Abbott). The anaesthetic was slowly injected into an ear vein. A tracheotomy was performed and mechanical ventilation was applied using a positive pressure respirator for small animals, at a rate of thirty-five respirations/min, in order to keep blood gases within the normal range. Two polyethylene catheters were inserted, one in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multichannel recorder (Nihon-Kohden, Model 6000, Japan) and the other one in the jugular vein for the intravenous drug administration. After the surgical preparation, no action was performed for 3-4 min in order to stabilise animals' blood pressure. During the subsequent experimental procedure no more anesthetic was given to animals.

***Group 0: Blind***

In the Blind experiment, four animals were continuously infused with dextrose 5% through a syringe pump. The rate of the infusion was 0.2 ml/min while each experiment lasted 20 min.

***Group I: Control***

Ang II was dissolved in dextrose 5% at a final concentration of 5 μg/ml. An infusion rate of 0.2 ml/min (1 μg/min) was used in order to induce substantial vasoconstriction and blood pressure increase. Ang II was infused continuously for a 20-min period via a syringe pump (Harvard Apparatus Pump 22, Harvard Apparatus, Natick, MA, USA). Blood pressure measurements were recorded both during the infusion period and the first three minutes of the recovery period when Ang II infusion was stopped.

***Group II (A&B): Effects of losartan and EXP 3174 administration during the Ang II infusion***

Animals in this group were subjected to continuous Ang II infusion as it was already described for group I. In group **$II_A$,** a single bolus dose of 0.2 mg/kg of either losartan **($II_{AL}$)** or EXP 3174 **($II_{AE}$)** was injected the 3rd min of the study, while in group **$II_B$**, losartan (**$II_{BL}$**) or EXP 3174 (**$II_{BE}$**) were given in a sequential manner of 0.2 mg/kg, 0.4 mg/kg and 0.4mg/kg the 3rd, 7th and 12th min of the study, respectively. Losartan was dissolved in 0.9% NaCl solution and EXP 3174 was dissolved in a 5% $NaHCO_3$/dextrose (50:50) solution.

***Group III (A&B): Study of losartan and EXP 3174 pre-treatment effects***

In Group $III_A$, animals were pre-treated with a single intravenous bolus dose of 0.2mg/kg losartan **($III_{AL}$)** or EXP 3174 **($III_{AE}$).** Ang II infusion started after a 4 min pre-treatment interval and lasted for a time period of 20 min. In order to study the pre-treatment dose dependence, the animals of Group $III_B$ were administered with a single intravenous bolus dose of 0.4mg/kg losartan **($III_{BL}$)** or EXP 3174 **($III_{BE}$)** while the rest of the experimental procedure was kept identical.

The procedure which was followed in each experiment is presented in Figure 1.

***Group IV: Study of synthetic compound effects***

In this group a non potent synthetic compound, *(5S)*-5-(1*H*-imidazol-1-ylmethyl)-1-(phenylmethyl)-2-pyrrolidinone (MM1), was used in order to validate the proposed protocol. [28] All the procedures followed in the losartan and EXP3174 including post – and pre – treatment were also applied to the synthetic compound.

***Statistical analysis***

Blood pressure measurement was represented by mean arterial pressure, MAP (mmHg). All variables are expressed as mean ± standard deviation (SD). Student's paired *t*-test was used to compare MAP values and differences between different time intervals of the study. One factor analysis of variance (ANOVA) was performed to determine the statistical significance of the variation of the MAP values between the control group and groups II, III and IV (study groups) at time intervals t=7, t=12, t=20. A *p* value less than 0.05, which rejects the null hypothesis, was considered as the level of statistical significance, indicating the effectiveness of the administered drug. Statistical analysis was performed on a SPSS 13 version (SPSS Inc., Chicago, IL, USA).

***Results***

The recorded MAP ± SD levels for all the experiments are presented in Table 1 and the associated graphs are depicted in Figures 2 and 3.

***Group 0*****:** The aim of this sub-experiment is to study any possible effect of the anesthetic drug in arterial blood pressure. Pentothal was used as anesthetic for surgical preparation and no further administration was performed during the recording period in order to not interfere with the results. No significant difference was found regarding MAP levels after pentothal administration compared with baseline levels (p=0.42)

***Group I:*** Group I actually served as a control group for all the other study groups. Continuous infusion with 1 μg/min Ang II induced significant vasoconstriction during the study intervals. The Ang II infusion produced initially a progressive increase of blood pressure which obtained its highest value at a time period of 3 min and was sustained until the 12$^{th}$ min of the study. Loss of responsiveness to Ang II was observed after the 12$^{th}$ min of the study since a significant decrease in MAP levels was revealed between time periods t=3 and t=20 min. The decrease in MAP could possibly be attributed to the desensitization to Ang II infusion which became pronounced after the 12$^{th}$ minute of continuing infusion of Ang II.

***Group II:*** Antihypertensive efficacy of losartan and EXP3174 in both groups, $II_A$ and $II_B$ was assessed by measuring the mean arterial pressure at specific time-intervals over twenty minutes.

**Effects of post–treatment with losartan:** The single bolus dose of 0.2mg/kg losartan administered the 3$^{rd}$ min of the study markedly attenuated the pressor response to Ang II. The maximum inhibitory effect was evident 4-5 min after the injection (p=0.01), t=7, Group $II_{AL}$, Fig.2). The efficiency of the drug was evident until the 7$^{th}$ min of the study, as the statistical analysis shows (p=0.012). From the 7$^{th}$ to the 20$^{th}$ min, no further decrease in MAP levels was revealed. Seeking to prolong the inhibitory time- interval of the drug, two more doses of losartan were injected at the 7$^{th}$ and 12$^{th}$ min. The dose for the second and third bolus was 0.4mg/kg each, as previously described. [28,29] However, as it was expected, sequential intravenous injections of losartan showed significant decrease in MAP levels

during the whole study period compared with baseline levels and single dose losartan administration (p= 0.045, Group $II_{BL}$, Fig.2).

**Effects of post–treatment with EXP 3174**: Compared to control group, single 0.2mg/kg EXP 3174 administration inhibited the pressor response to Ang II. Specifically, normal MAP levels were evident 4-5 min after the injection and the inhibitory effect remained significant until the end of the experiment (p = 0.06, Group $II_{AE,}$ Fig. 2). When intravenous injections of EXP 3174 were given at a sequence of 0.2, 0.4 and 0.4 mg/kg, the MAP lowered to normal levels from the initial dose of 0.2 mg/kg and remained within normal values until the end of the study, as it was expected (p= 0.02, Group $II_{BE,}$ Fig. 2). Although EXP 3174, like losartan, presented a maximal inhibitory effect 4-5 min after the injection, it showed a significant decrease in MAP which was sustained until the end of the study (t=20), regardless of the drug protocol (single *vs.* three doses).

***Group III***: Pre-treatment with losartan and EXP 3174 (0.2mg/kg for Group $III_A$ or 0.4mg/kg for Group $III_B$) had no influence on baseline MAP levels during the pre-treatment period [t= -4 to t=0 min, losartan 0.2mg/kg (p=0.18), losartan 0.4mg/kg (p=0.10), EXP 3174 0.2mg/kg (p=0.75) and EXP 3174 0.4 mg/kg (p=0.33)].

**Effects of pre-treatment with losartan:** Pre-treatment with losartan (0.2mg/kg) does not prevent MAP from reaching its maximal value following infusion of Ang II (t=0) compared with the control (p =0.016, t=3, Group $III_{AL}$, Fig. 3). The inhibitory efficiency of the drug is evident during the $7^{th}$ min after Ang II infusion. The profile of losartan efficiency parallels the one of the post-treatment procedure. However, duplication of the pre-treatment dose of losartan results in a different profile. The vasoconstrictor response to Ang II is significantly reduced as it is shown in Fig. 3. Use of a higher dose of losartan causes higher inhibitory effect, and even a longer duration of action (until the $12^{th}$ minute of the study) as it is depicted by its more pronounced MAP lowering. This indicates that losartan causes a dose-dependent inhibitory effect. From the $12^{th}$ until the $20^{th}$ minute of the study, the efficiency of the drug is not increased.

**Effects of pre-treatment with EXP 3174:** Pre-treatment with EXP 3174 highlights the greater potency of the active metabolite of losartan. The administration of 0.2 mg/kg induced a more effective blockade of the receptor, showing a greater attenuation of maximal pressor response to Ang II

compared to the same dose of losartan administration (Fig. 3). On the contrary, the duration of action of 0.2 mg/kg EXP3174 was the same as that of losartan, since significant MAP variations between group $III_{AE}$ and control group were observed until the $7^{th}$ min ($p=0.01$). When the pre-treatment dose was doubled, EXP3174 showed inhibition of the pressor response of Ang II in a dose-dependent manner as mentioned with losartan. Although, pre–treatment with 0.4 mg/kg EXP 3174 showed quite the same blockade with 0.4 mg/kg losartan, the duration of action was increased for EXP 3174 since it was sustained during the whole study period.

**Validation study:** The pressor response of Ang II on MAP were unchanged any time after MM1 administration either in the post or in the pre treatment mode of Ang II infusion as the statistical analysis shown. Out results confirm the success of the protocol in screening novel synthetic molecules for being potential drugs since it is known that MM1 is a compound with a very low affinity at $AT_1$ receptors in vitro. [29]

**Discussion**

The main findings of the present study underline for the first time that continuous infusion of Ang II at a dose of 1 μg/min for 20 minutes in anaesthetized rabbits, represents a rapid and safe pharmacological screening test for $AT_1$ antagonist.

All the animals were anaesthetized in order to avoid any interference of the stress to the results. Furthermore, no additional anesthetic was given during mean arterial pressure (MAP) recordings and therefore a potential undesirable drug interaction was avoided. The claims for general anesthesia confirm that a 20 minutes interval is safe and devoid stress reactions.

In the first step of the study, we tested the effects of the continuous Ang II infusion at a dose of 1 μg/min. Previous studies have reported that there is an hypertensive response to Ang II infusion at a higher concentration than 0.5 nmol/kg whilst the elevation of mean arterial pressure (MAP) with lower doses of Ang II is easily overcome from the depressor response that usually follows. [30] Gavras and colleagues suggest that Ang II infusion in rabbits at a dose of 1.4 μg/kg/min, leads to increased concentration of Ang II plasma levels that is slightly higher than the observed in humans with malignant hypertension. [31] Based on previous studies, [32] we chose the continuous infusion of Ang II at a dose of 1 μg/min in order to induce substantial elevation in MAP and then to evaluate the effectiveness of the drugs under examination on blood pressure response. The infusion rate in our study increased the MAP from 100±9 mmHg to 172±6 mmHg. This was the maximal response that was obtained three minutes after the start of infusion. A stable interval lasting until the 12th min was followed and then loss of response to Ang II was recorded. The rapid desensitization to Ang II infusion is a known effect but the exact time-interval depends on the concentration of infused Ang II and the animal model. Nielsen and colleagues have reported that the continuous infusion of Ang II in conscious rabbits in an identical concentration, which actually was the same with the one we used, caused a blood pressure elevation of 70 mmHg. [33] This effect was transient and the blood pressure returned to baseline values one to two hours after the initiation of Ang II infusion. Those findings confirm that a 20 minutes study should be ideal in order to obtain safe results regarding the effectiveness of studied compounds.

Next in our study, we compared the efficacy of the drugs under investigation regarding blood pressure decrease using two methods of intervention, one post-treatment and one pre-treatment. This comparison provides additional information regarding the treatment regime that may be beneficial in terms of duration of action and efficacy of the drug as well as the appropriate concentration. Post-treatment experiments were carried out using either a single or three sequential injections of the drug molecules. The first dose was 0.2 mg/kg, and it was chosen based on previous *in vivo* studies in which it was well tolerated. [34] A comparison between single and sequential dosage revealed that there was a drug-dependent correlation. Using the single dose protocol with losartan, a significant lowering of the BP was maintained up to the 7$^{th}$ min. Mean arterial pressure levels did not significantly differ after the 12$^{th}$ min compared to the control, probably due to the acute desensitization of Ang II. Although, a further decrease in mean blood pressure was expected in the sequential protocol, this was not observed and the reason is not clear. However, it is possible that the study period was not suitable for the metabolism of the entire drug amount. The above results suggest that the sequential manner of administration is not an appropriate methodology for a dose-depended evaluation of the study compound.

EXP3174 decreased the mean arterial pressure to the normal levels even from the first chosen dose of 0.2 mg/kg. In the single dose protocol, the duration of EXP 3174 action was sustained from the 7$^{th}$ min of the infusion up to the end of the study. The same pattern observed when three sequential doses were used and normal mean arterial pressure was observed from the first dose with no additional change after the 2$^{nd}$ and 3$^{rd}$ injection. These findings suggest that the use of a single dose protocol is sufficient to provide a safe intervention with the molecule under investigation in the post-treatment arm of the study. The maximal inhibitory effect of both molecules appeared after the 5$^{th}$ minute of the study, a time point that there is no evidence of desensitization to Ang II. In many previous reports the maximal inhibitory effect of losartan or EXP3174 was evident at 5 min post injection regardless the dose, the experiment procedure or the animal model which was used. [13,17] This may suggest that the 20 minutes study period of our protocol is a safe interval for the evaluation of a novel $AT_1$ antagonist.

The final series of experiments includes the study of a pre–treatment methodology. The infusion of either losartan or EXP 3174 in each of the administered dose of 0.2 or 0.4 mg/kg had no effect, indicating that the activated RAAS system is significant for the expression of the activity. This was not unexpected as a great number of evidence from both in vivo experimental studies and clinical studies in humans resulted to the conclusion that an $AT_1$ antagonist requires an activated RAAS in order to demonstrate its blood pressure lowering effect in normotensive subjects. [13,19] This may be attributed to $AT_1$ receptor activation through Ang II binding which can induce conformational change of the receptor.

In the pre-treatment methodology, both losartan and its metabolite EXP 3174 inhibit the response to Ang II infusion. However, they demonstrated a different profile of $AT_1$ receptor blockade even if they were administered in the same dose. When a low dose (0.2 mg/kg) was used, the duration of the action for both drugs was similar but the maximum mean arterial pressure response was different. EXP 3174 induced a remarkable lowering of the maximum blood pressure, after Ang II administration, while losartan treatment did not cause a similar effect. By duplicating the dose of losartan and EXP 3174 to 0.4 mg/kg, the duration of action was longer, an observation which is consistent with previous reports. [35] As it was expected 0.4 mg/kg of losartan compared to 0.2 mg/kg resulted in further reduction of the maximum blood pressure response to Ang II infusion.

Pre-treatment with an insurmountable antagonist may actually prevent Ang II binding to the active site resulting in lowering of the maximum blood pressure response. This finding is not in agreement with the high dose pre-treatment study, where both losartan and EXP 3174 reduced the maximum blood pressure response. Nevertheless our results may indicate that the competitive or non competitive nature of an $AT_1$ antagonist is possibly related to the concentration of the drug at least in the pre-treatment procedure.

Both post– and pre–treatment procedures can be used as fast screening methods for $AT_1$ antagonists. Our protocol enables the evaluation of $AT_1$ antagonists using lower doses (0.2mg/kg) than those reported by other scientific protocols. The sequential methodology seems unable to give evidence about the dose-dependence correlation and separate experiments with different

concentrations should carry out in order to extract a dose-dependent correlation as emerges from pre-treatment experiments.

The use of anaesthetized models effectively minimizes the impact of the produced stress that makes inaccurate the recording of true blood pressure levels. Moreover the fact that the animals we used for the study did not recovered, not only alleviates the animals from pain but also avoid acute BP increase, a fact that follows the restrained animals. Furthermore, the use of a direct method for blood pressure recording enables the valuable continuous quantitative blood pressure estimation. Advantage of our protocol is that it is not expensive, permitting almost any laboratory to use it. More specifically, since we used a short term protocol in healthy animals, there was no reason to keep and breed them for a long time period. Additionally, only a small amount of the studied molecules is needed for safe and convincing results. This is a worth point to mention, as in early drug development the amounts of the compounds synthesized are limited.

The developed fast protocol was validated using the synthetic, almost inactive MM1 compound which was designed and synthesized so as to mimic the $His^6$-$Pro^7$-$Phe^8$ part of Ang II and is based on the (S)-pyroglutamic acid. The results confirmed previous findings *in vitro*, indicating that the applied protocol can differentiate the active from the inactive compounds *in vivo*, in a very short time period.

In conclusion, the protocol which we propose appears to be safe and effective for the pharmacology screening of novel $AT_1$ antagonists. Continuous infusion with Ang II in anaesthetized rabbits enables the evaluation of $AT_1$ antagonists using very low doses of the tested drug. Moreover, this protocol is characterized by its simplicity and rapid accomplishment.

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*Figure legends*

**Figure 1:** Schematic representation of the experimental groups showing infusion sequences and time duration of the experiment.

**Figure 2:** A time course of mean arterial pressure measurements (MAP)±SD due to continuous intravenous infusion of Ang II in control and post-treatment groups.

**Figure 3:** A time course of mean arterial pressure measurements (MAP)±SD due to continuous intravenous infusion of Ang II in control and pre-treatment groups.

**Table 1:** Mean arterial pressure levels recordings in all groups both in post-treatment and pre-treatment experimental procedures.

| | MAP ± SD (mmHg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Time / Group** | **t= -4** | **t= -2** | **t =0** | **t= 3** | **t= 7** | **t= 12** | **t =20** | **Ang II infusion termination** |
| | | | | **Vehicle Groups** | | | | |
| **Blind** | | | 123±7 | 122±7 | 120±9 | 115±12 | 118±14 | |
| **Control** | | | 100±9 | 172±6 | 165±6 | 154±7 | 145±13 | 100±25 |
| | | | | **Post – treatment Groups** | | | | |
| **Group $II_{AL}$** | | | 112±8 | 174±21 | 127±20 | 128±20 | 128±15 | 98±9 |
| **Group $II_{AE}$** | | | 109±15 | 182±31 | 97±17 | 99±13 | 104±4 | 87±2 |
| **Group $II_{BL}$** | | | 88±32 | 175±12 | 145±8 | 130±6 | 114±19 | 88±25 |
| **Group $II_{BE}$** | | | 105±22 | 171±15 | 81±18 | 74±13 | 81±13 | 81±12 |
| **Group $IV_{A}$** | | | 135±19 | 187±18 | 175±18 | 160±5 | 157±4 | 116±13 |
| **Group $IV_{B}$** | | | 103±29 | 162±19 | 149±13 | 141±16 | 134±13 | 103±18 |
| | | | | **Pre – treatment Groups** | | | | |
| **Group $III_{AL}$** | 117±8 | 114±8 | 108±9 | 173±9 | 150±5 | 140±16 | 128±11 | 98±10 |
| **Group $III_{AE}$** | 124±32 | 119±30 | 117±30 | 151±9 | 142±12 | 138±13 | 137±12 | 109±20 |
| **Group $III_{BL}$** | 120±9 | 117±11 | 107±12 | 132±18 | 135±10 | 137±10 | 135±9 | 102±15 |
| **Group $III_{BE}$** | 101±34 | 100±35 | 94±28 | 114±25 | 116±11 | 106±26 | 112±18 | 92±18 |
| **Group $IV_{A}$** | 116±9 | 118±9 | 113±9 | 183±10 | 167±11 | 152±9 | 144±8 | 103±13 |
| **Group $IV_{B}$** | 129±12 | 132±7 | 125±5 | 193±5 | 167±10 | 148±12 | 144±16 | 102±5 |

MAP: mean arterial pressure, SD: standard deviation

**Figure 1**

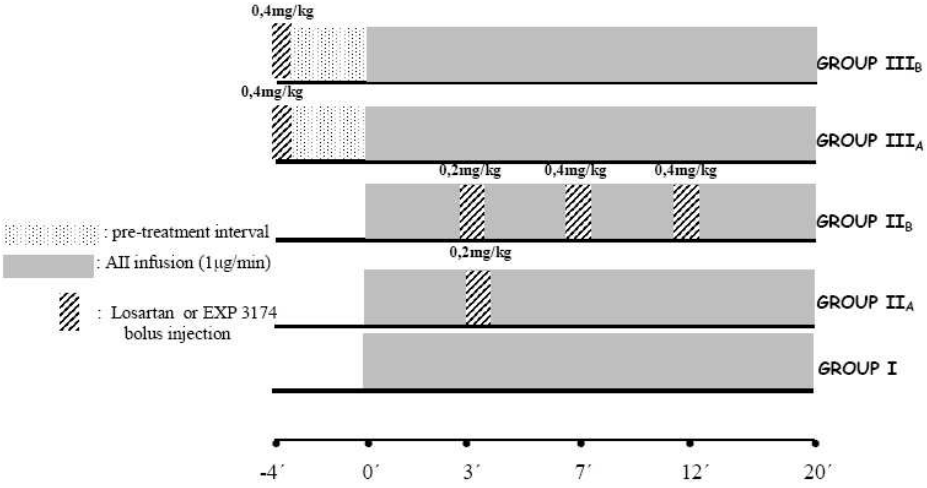


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**Figure 2:**

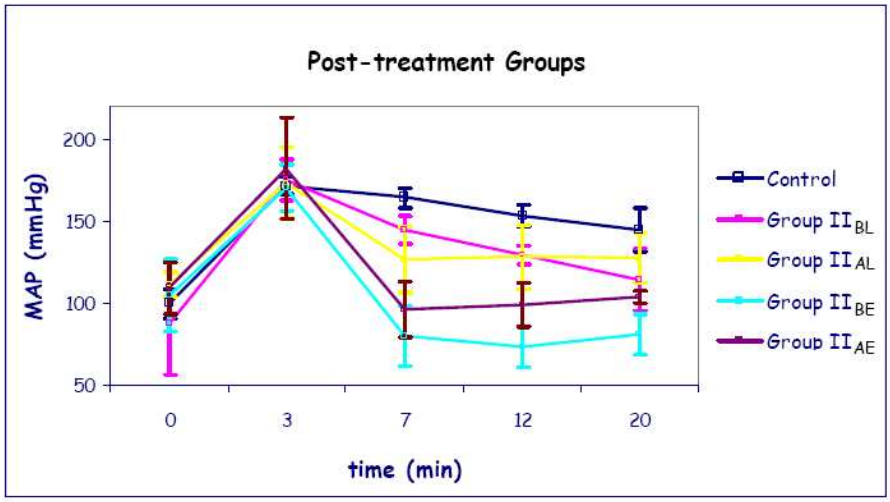


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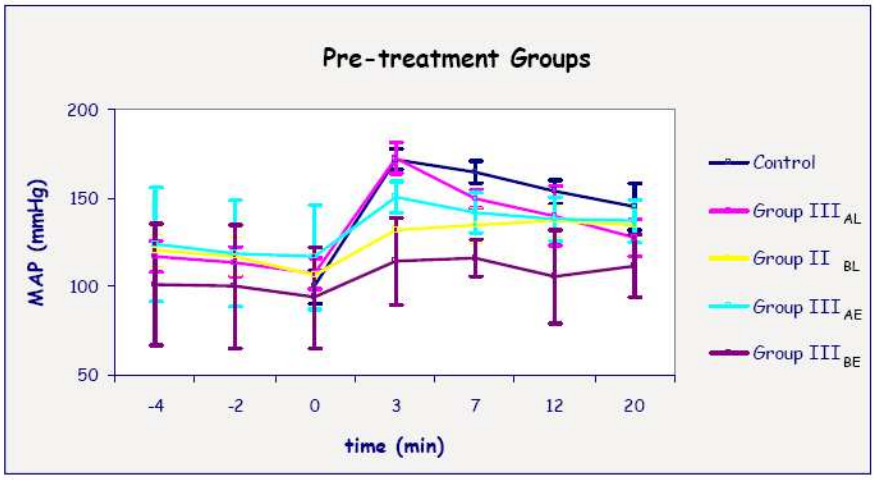
**Figure 3:**

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