Suppression of Fas expression in hypertensive placental histology given Nano herbal of Rhodomyrtus tomentosa leaves

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Suppression of Fas expression in hypertensive placental histology given Nano herbal of *Rhodomyrtus tomentosa* leaves

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Abstract

High blood pressure during pregnancy can signal a high-risk pregnancy and result in difficulties. The expression of Fas is critical for maintaining hypertensive immunity. Fas is prevalent in the trophoblast, which enhances maternal-derived apoptosis. Because it contains acylphloroglucinol, flavonoids, tannins, and triterpenes, Rhodomyrtus tomentosa (haramonting) is commonly used in traditional Indonesian medicine to treat high blood pressure. The goal was to determine and investigate the role of haramonting in lowering Fas expression in hypertensive rats' placental histopathology. The rats were confirmed to be pregnant were divided into five treatments: (a) normal pregnant rats (control), (b) hypertensive rats (c) hypertensive rats + 100mg/KgBW NRT, (d) hypertensive rats + 200mg/KgBW NRT, and (e) hypertensive rats + 400mg/KgBW NRT. On gestation day 20, pregnant rats were euthanized under ketamine anaesthesia. ELISA was used to examine the expression of the HSP family. Immunohistochemistry was used to assess Fas expression. In hypertensive rats, NRT can lower systolic and diastolic blood pressure ($P<0.01$), and haramonting can improve placental efficiency and restore birth weight. In hypertensive rats, the higher the dose of NRT, the higher the levels of HSP27, HSP70, and HSP90. In placental histology, NRT suppresses Fas expression as anti-apoptotic in trophoblast cells. Because NRT is high in antioxidants and protects cells from hypoxia and dehydration, it suppresses Fas activity in the labyrinthine zone, basal zone, and Yolk Sac.

Keywords: Fas; herbal medicines;hypertensive; Immunohistochemistry;HSP

1. Introduction

Hypertension is considered a marker of the emergence of chronic inflammatory diseases. Hypertension or high blood pressure is one indicator of a high-risk pregnancy. Hypertension in pregnancy can be mild, but if it is not handled properly it can cause serious problems and even threaten the life of both the mother and the fetus (Li *et al*., 2011). If chronic hypertension is not treated effectively during pregnancy, it might develop to chronic hypertension and preeclampsia. Protein in the urine is a symptom of this illness, which can lead to complications like congestive heart failure, visual abnormalities, stroke, seizures, and kidney or liver disorders (McLaughlin *et al*., 2018).

Fas is a glycoprotein that causes apoptosis and is involved in the maintenance of tumor immunity as well as hypertension (Iqubal *et al*., 2018). Fas was shown to be a crucial molecule in the immune system's death signal transduction and to trigger apoptosis in Fas-positive cells by binding FasL to the extracellular domain of Fas. (Ramezani *et al*., 2019). In the LPS animal model, activation of the Fas system and induction of apoptosis can cause placental abnormalities (Ejima *et al*.,2000). The extravillous trophoblast and the syncytiotrophoblast layer, the major site of the feto-maternal interface, display consistent immunoreactivity to Fas in the placenta (Yougbaré *et al*., 2017). FasL's distribution pattern is nearly identical to that of Fas and Bcl-2 (Yougbaré *et al*., 2017). Fas is prevalent in the trophoblast, which contributes to immunity and causes apoptosis in maternally derived activated Fas-expressing cells. The fas pathway can trigger apoptosis in placental cells during implantation (Ejima *et al*., 2000).

Oxidants can regulate Fas-mediated cell death. Both pharmacological antioxidants, and antioxidant enzymes. Fas signaling induces ROS production. Fas-induced activation of caspase-8 may be a target for redox regulation (Benhar, 2020). Thiol antioxidants can inhibit Fas-mediated apoptosis in B-cell lymphoma cell lines. Thus, it is known that Fas-stimulated ROS generation plays a key role in Fas-mediated apoptosis (Benhar, 2020). ROS generation was detected in cells sensitive to apoptosis but not in those inherently resistant to anti-Fas. In Fas-sensitive cells, there is depletion or antagonism in endogenous antioxidant defenses in increasing sensitivity to Fas-induced apoptosis (Devadas *et al*., 2003). Mechanistic studies show that exogenously and endogenously generated ROS function to promote the activation of initiator caspases such as caspase-8 (Devadas *et al*., 2003). Thus, it was found that the level of oxidative stress, either from exogenous sources or endogenously generated on receptor stimulation, regulates the sensitivity of tumor cells or hypertensive cells undergoing Fas-mediated apoptosis.

The oxidant used in this study was an antioxidant from *Rhodomyrtus tomentosa* leaves. Indonesian people call it haramonting. This plant is often used in traditional Indonesian medicine to treat colic diarrhea, dysentery, abscesses, bleeding, high blood pressure drugs, and gynecopathy. Extracts from this plant contain acylphloroglucinol, flavonoids, tannins, and triterpenes (Vo & Ngo, 2019). Haramonting leaves have antioxidant activity in vitro and in vivo. This plant can also inhibit lipid peroxidation, improve the histology of the placenta, testes and lungs of smokers, increase the expression of HSP-70 and increase the ability to reduce free radicals (Ilyas, S & Situmorang, 2021; Ilyas *et al*., 2020; Situmorang et al., 2018a; Manurung *et al*., 2021; Irianti *et al*., 2020). The presence of high amounts of phenolic compounds results in a high antioxidant capacity so that it has the potential as a source of health-promoting compounds (Zhang *et al*., 2018). The purpose of this study was to determine and analyze the role of haramonting in suppressing the expression of Fas in the histology of the placenta of hypertensive rats before continuing to use human cells. Haramonting was converted to nano size in order to reduce the shape so that it has good penetration and bioalvability in cells (Lee *et al*., 2017). It is hoped that this plant can be developed for molecular hypertension therapy drugs in humans and pregnant women.

2. Materials and Methods

2.1 Materials

Haramonting (*Rhodomyrtus tomentosa*) was obtained from Rantauprapat city, Labuhanbatu North Sumatera Province. Nano herbal *Rodomyrtus tomentosa* (NRT) was carried out at the Indonesian Research Institute (LIPI) in Jakarta, Indonesia by *High-energy milling* (HEM) (Situmorang & Ilyas, 2018a;Ilyas *et al*.,2020; Situmorang *et al*.,2021a). Intraperitoneal administration of LPS within 3 hours by means of Escherichia coli 026:B6 (1 mg/kg BW) dissolved in 0.9% NaCl. Ketamine hydrochloride (30 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ, USA) is used as an anaesthetic before surgery. Santa Cruz Biotechnology provided anti-Fas (sc-715) antibodies (SanverTech, Heerhugowaard, the Netherlands). Cell Signaling Inc. is a company providing the mouse anti-phosphotyrosine and rabbit anti-Hsp27, anti-Hsp70, and anti-Hsp90 antibodies (Danvers, MA, USA) in our study.

2.2 Animal Handling

The experimental animals used were pregnant female Wistar rats with a weight of 150-200g and 2 months old in healthy condition. This research has passed the animal ethics review from the Ethics Commission of the Faculty of Mathematics and Natural Sciences,USU (Ethical Clearance: No. 0259/KEPH-FMIPA/2021). In an air-conditioned room with a 12-hour light/dark cycle, the animals were fed conventional laboratory diets and given free access to water. A pair of Wistar rats were mated overnight and observed using the CCTV camera provided in the cage. The pregnancy of female rats was confirmed by vaginal plug and the day was declared as day 0. In pregnant rat on gestational day 6, systemic inflammation was produced by infusing Escherichia coli lipopolysaccharide (LPS) into the peritoneal cavity using a small osmotic pump. Before surgery, the animals were sedated with ketamine on the 19th day of pregnancy.

2.3 Design of experimental

The rats were confirmed to be pregnant were divided into five treatments: (a) normal pregnant rats (control), (b) hypertensive rats (c) hypertensive rats + 100mg/KgBW NRT, (d) hypertensive rats + 200mg/KgBW NRT, and (e) hypertensive rats + 400mg/KgBW NRT. Pregnant rats were excised on gestation day 20 under ketamine anesthesia. The markers of hypertension was characterized by a measure of blood pressure, and HSP family expression analyzed by ELISA. Expression of Fas protein was evaluated by immunohistochemistry.

2.4 Measurement of systolic and diastolic blood pressure in pregnant rats

Blood pressure was determined before LPS injection, after LPS injection and NRT administration. Systolic and diastolic arterial blood pressures were recorded in conscious and uncontrolled animals for 1 hour and mean arterial blood pressures using the non-invasive tail cuff method.

2.5 ELISA

The ELISA test from Enzo Life Sciences was used to perform quantitative HSP analysis. The HSP27 (rabbit) ELISA kit, the HSP70 high-sensitivity ELISA kit, and the HSP90 (rabbit) ELISA kit were utilized in this study. According to the manufacturer's recommendations, each sample was performed three times.

2.6 Hematoxylin-eosin (H&E) staining

The placenta was placed in xylol for 15 minutes, after which the tissues were then alternately hydrated in 96% and 70% pure alcohol for 5 minutes, respectively, then rinsed lastly in distilled water. Hematoxylin dye was then applied for 5 minutes, then rinsed last 3 minutes in distilled water. Add eosin dye for 1 minute. The slides were dehydrated with 70%, 96% and 100% alcohol. The slides were immersed in xylol and put on a cover glass. The sample is examined under a light microscope with 5 times the field of view (Irianti *et al.*, 2020).

2.6 Immunohistochemistry

To suppress endogenous peroxidase activity, 5 m thick paraffin-embedded placental slices were deparaffinized and treated with 1 percent H2O2 in methanol for 30 minutes before immunohistochemistry. After that, the slides were rinsed in 0.01 M Tris-buffered saline (TBS pH 7.4). Tissue slices were treated with primary anti-Fas antibody (1:100 dilution in PBS containing 1 percent BSA) for 2 hours after blocking non-specific binding to 1 percent skimmed milk in PBS. The Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used to identify immunoreactivity, which was counterstained with Mayer's hematoxylin.

2.7 Statistic analysis

Research data Blood pressure and HSP family were analyzed using one-way ANOVA and immunohistochemistry ad tunnel assay using Kruskal Wallis on Sigmaplot software. Value was analyzed with mean and standard deviation.

3. Results and discussion

3.1 Role of Nano herbal *Rhodomyrtus tomentosa* (NRT) on Systolic and diastolic blood pressures

Table 1. The effects of hypertension on systolic and diastolic blood pressures with and without maternal NRT treatment

Groups	Systolic blood pressures			Diastolic blood pressures		
	Day 0	Day 6	Day 19	Day 0	Day 6	Day 19
C-	121 ± 8.02	122 ± 9.12	120 ± 5.99	85 ± 2.34	90 ± 4.90	87 ± 3.99
C+	122 ± 7.71	146 ± 9.23*	150 ± 6.76**	90 ± 4.01	118 ± 3.98 *	120 ± 6.98**
NRT100	122 ± 4.54	146 ± 3.56*	136 ± 7.55*	89 ± 4.91	115 ± 5.88*	115 ± 5.87*
NRT200	123 ± 5.98	150 ± 4.98**	130 ± 6.89*	87 ± 4.89	116 ± 5.99*	100 ± 5.89*
NRT400	121 ± 7.78	145 ±.7.22*	127 ± 6.22	88 ± 8,21	115 ± 7.04*	95 ± 4.76

Note: C-: Untreated, C+: Hypertension rat, NRT100: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, NRT200: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, NRT400: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW. Day 0: Before LPS Injection, Day 6: After LPS Injection, Day 19: After administration of Nano herbal *Rhodomyrtus tomentosa* (NRT). (*P<0.05, **P<0.01 compared C- group).

From the lowest to the highest dose, NRT can lower blood pressure. There was no significant difference between all treatments (P>0.05) on day 0 since all animals had normal systolic and diastolic blood pressure. On the sixth day, all groups except group C- were given LPS injections, and there was a significant increase in systolic and diastolic blood pressure (P<0.01) compared to group C-. NRT was given to hypertensive rats for a week from the 13th to the

19th gestational days and was found to lower systolic and diastolic blood pressure (P<0.01). This research supports Irianti *et al* (2020)'s findings that haramonting can lower blood pressure in preeclampsia pregnant rats while also increasing trophoblast count and placental histology.

3.2 Effects of Nano herbal *Rhodomyrtus tomentosa* (NRT) treatment on birth weight

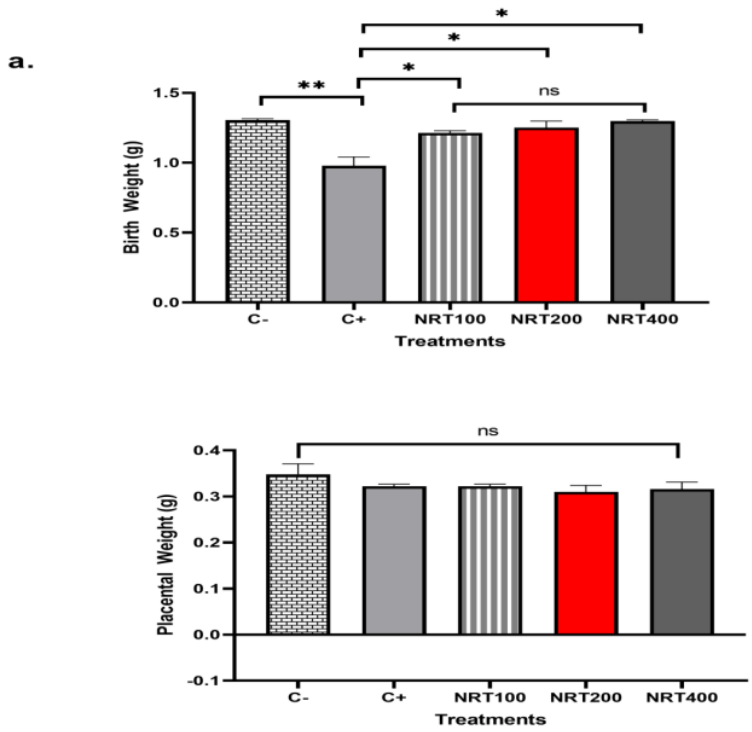

Fig. 1. Effects of Nano herbal *Rhodomyrtus tomentosa* (NRT) treatment on fetal and placental weight. a.Birth Weight, b. Placental weight. C-: Untreated, C+: Hypertension rat, NRT100: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, NRT200: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, NRT400: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW.). (^{ns}P>0.05, *P<0.05, **P<0.01 compared C- group).

The NTR treatment resulted in a significant difference in the birth weight of hypertensive rats (P<0.01). Hypertension can lead to fetal weight loss and disrupt the passage of nutrients from the mother to the fetus. However, administration of NRT at various doses, from the lowest to the greatest, has revealed that this herb can restore fetal weight in hypertension (P<0.5) (Fig.1a). It had no effect on placental weight in either healthy, hypertensive, or NRT treatments (Fig1.b). Pregnancy problems include placental insufficiency, hypertension, and preeclampsia cause the transport of nutrients to the fetus to slow down. Reduced placental and umbilical blood flow is to blame (Meah *et al*., 2020). Therefore, Intrauterine growth retardation, caused by reduced nutrition delivery to the fetus during pregnancy, is a significant health burden due to perinatal morbidity and mortality (Meah *et al*., 2020). One of the causes of the transportation of nutrients and oxygen is the delayed growth of the fetus due to the development of cardiovascular and metabolic illnesses, or hypertension (Thornburg *et al*., 2010).

Antioxidants can help to promote placental function while also lowering intrauterine growth. Because NRT contains very strong antioxidants, it can overcome this (Situmorang et al., 2021b). Oxidative stress in the placenta and fetus may be exacerbated by a lack of nutrients and oxygen (Wu *et al*., 2012). The findings of this study confirm this theory, indicating that treating gestational hypertension with haramonting can increase placental efficiency and restore birth weight.

3.3 Effects of Nano herbal *Rhodomyrtus tomentosa* (NRT) in Expression of HSP27, HSP70 and HSP90

There was a substantial difference in HSP27 levels in hypertensive rats with the NTR therapy ($P<0.05$). The highest NRT dose resulted in a greater difference ($P<0.01$). In hypertensive rats, the higher the dose of NRT, the higher the dose of HSP27. NRT treatment induced high levels of Hsp27 expression because it acts as a molecular chaperone, regulating the folding and renaturation of damaged proteins, which can happen after LPS and oxygen free radicals are injected. HSP27 also has a role in physiological reactions to body fluid homeostasis, which has an impact on blood pressure regulating systems. (Gostimirovic *et al*., 2020).

HSP70 levels in hypertensive rats treated with NTR at dosages of 100 to 400 mg/kg BW showed a significant difference ($P<0.05$). Hsps act as antiapoptotic agents via regulating caspase activation, C-jun NH2-terminal kinase activation, and the nuclear factor-B (NF-B) pathway activation (Iordanov *et al*., 2000). Hsp70 expression improves postischemic ventricular recovery, although overexpression of Hsp70 in transgenic rat improves postischemic cardiac function and reduces neuronal cell death (Gostimirovic *et al*., 2020). LPS-induced protection in hypertension and inflammation is due in part to suppression of NF-kB activation and lymphocyte activation. Lipid peroxidation, superoxide and hydroxyl radical anion activity can be prevented and countered by the antioxidant action of Haramonting (Situmorang *et al*., 2021b). Flavonoids in this plant extract can boost SOD and GSH-Px activity while also lowering MDA levels (Wu *et al*., 2015).

HSP90 levels in hypertensive rats dropped and rose ($P<0.05$) in response to NRT therapy. There was no significant difference ($P>0.05$) between doses of excellent NRT at doses of 100 to 400 mg/kg BW because any dose enhanced HSP90 levels, hence there was no difference in determining the best dose. HSP90 is a key player in the signaling pathway that leads to eNOS activation. HSP90 regulates vasomotor activity in resistance vessels by inhibiting Hsp90 signaling, which plays a role in modulating vasoactive responses in blood vessels (Jones *et al*., 2011). Increased HSP90 signaling promotes NO-dependent vascular hyporeactivity, indicating a link between protein-protein interactions in hypertension (Ai *et al*., 2003).

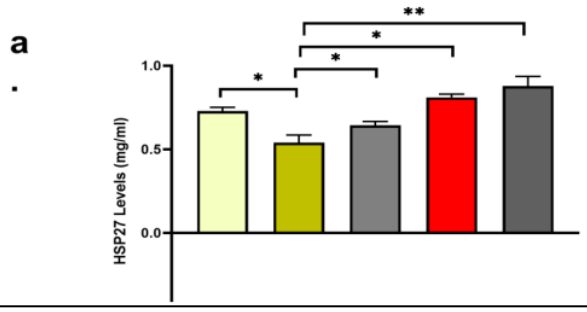

Fig 2. Effects of Nano herbal *Rhodomyrtus tomentosa* (NRT) in Head Shock protein family, a. HSP27, b. HSP70, c.HSP90. C-: Untreated, C+: Hypertension rat, NRT100: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, NRT200: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, NRT400: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW. ($^{ns}P>0.05$, $*P<0.05$, $**P<0.01$ compared C- group).

3.4 Fas expressions after given Nano herbal *Rhodomyrtus tomentosa* (NRT) to placental rats

According to the findings of the histopathological examination (Fig. 3), an increase in the number of syncytial nodes and in all placental tissue preparations may be present in the mature placenta (Fig. 3a), but syncytial nodes are present in the hypertensive placenta (Fig. 3b) due to the hypoxic situation experienced by the fetus placenta. Hypoxia causes the villi's ends or terminals to become irregularly shaped, increasing the likelihood of trophoblast accumulation and the creation of a syncytial knot. A shortage of oxygen perfusion causes an increase in cytotrophoblast. In the control placenta and at the NRT400 dose, mature cytotrophoblast cells with normal shape are formed, but in the histology of hypertension, many new villi are formed to support the lack of placental perfusion. The more trophoblast cells and new villi are formed, the more trophoblast cells and new villi are formed. Inadequate oxygen

perfusion causes trophoblasts to expand and become cytotrophoblasts. The function of the cytotrophoblast itself is the site of gas exchange where the cytotrophoblast will replace the endothelial function of the arterioles (Staff *et al*., 2020).

The absence or partial invasion of the spiral arteries by trophoblast cells causes vascular anomalies in hypertensive placentas (Brosens *et al*., 2019). The invasion involves the replacement of musculo-elastic spiral artery walls with fibrinoid-filled walls. However, the administration of NRT from the lowest to the highest dose (Fig.3c-e), began to improve these vascular abnormalities.

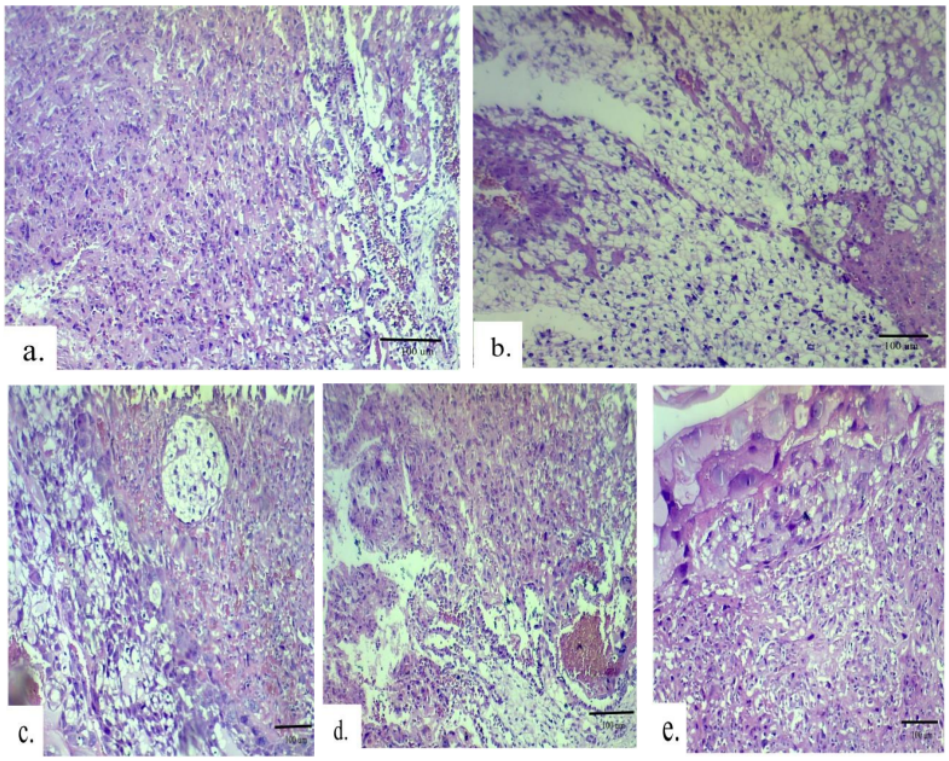

Fig 3. Histology of placental hypertension by Nano herbal *Rhodomyrtus tomentosa* (NRT) a. Untreated, b. Hypertension rat, c. Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, d. Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, e: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW (10x).

With NTR treatments, there was a significant difference in Fas expression in hypertensive rats ($P<0.05$). The highest NRT dose resulted in a greater difference ($P<0.01$). The lower the Fas activity of hypertensive rats, the higher the NRT dose (Table 2). In trophoblast cells, NRT suppression of Fas expression in placental histopathology may be anti-apoptotic (Fig.4). In humans, trophoblast tissue is involved in the regulation of maternal-fetal gas, nutrition, and waste product exchange. The labyrinthine zone is where maternal fetal exchange takes place in rats, while the basal zone is where placental hormone production takes place. The basal zone shows an increase in Fas expression in the hypertensive state but decreases with an increase in the dose of NRT (Fig.4). Caspase-8 may not be involved in the rise in Fas in the histology of hypertension. The intrinsic apoptotic pathway can mediate

increased caspase-3 and PARP levels (Staff *et al*., 2020). However, Further research into the mechanism causing apoptosis-induced cell death in the basal zone is needed. The labyrinthine zone of hypertension histology, in contrast to the basal zone, reveals elevated expression of all Fas pathway-related proteins that activate death via caspase-8(Staff *et al*., 2020). Yolk Sac had the highest Fas expression in placental histopathology in our investigation. Yolk Sac is a nutrient transfer system that takes nutrients from the uterine gland secretions or the mother's blood. The most eutherian Yolk Sacs lose contact with the peripheral chorion ontogenetically, forming free splanknopleuric Yolk Sacs that transport chemicals from the exocoelomic cavity (Zhao *et al*., 2018). Embryonic deformities, miscarriages, and growth problems can all be caused by errors in the Yolk Sac's development and function. In the labyrinth zone, basal zone, and Yolk Sac, NRT can decrease Fas activity (Table 2 and Fig.4). This is due to the presence of antioxidants such as anthocyanins, acylphloroglucinol, flavonoids, tannins, and triterpenes in NRT, which protect cells from hypoxia and death (Situmorang *et al*., 2021b).

Table 2. Fas expression in histology of hypertension placental

Treatment	Labyrinth zone	Labyrinth zone	Yolk salc
C-	12 ± 4.21	13± 5.89	20 ± 2.44
C+	32 ± 5.44$^{\#}$	30 ± 4.21$^{\#}$	61 ± 4.43**
NRT100	22± 9.61*	21± 5.22*	31± 4.91**
NRT200	20± 9.63*	19± 8.04*	22 ± 5.70*
NRT400	14± 3.28**	13± 7.32**	15± 3.53***

Note: C-: Untreated, C+: Hypertension rat, NRT100: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, NRT200: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, NRT400: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW. ($^{\#}$P<0.05 compared C- group,*P<0.05, **P<0.01 compared C+group).

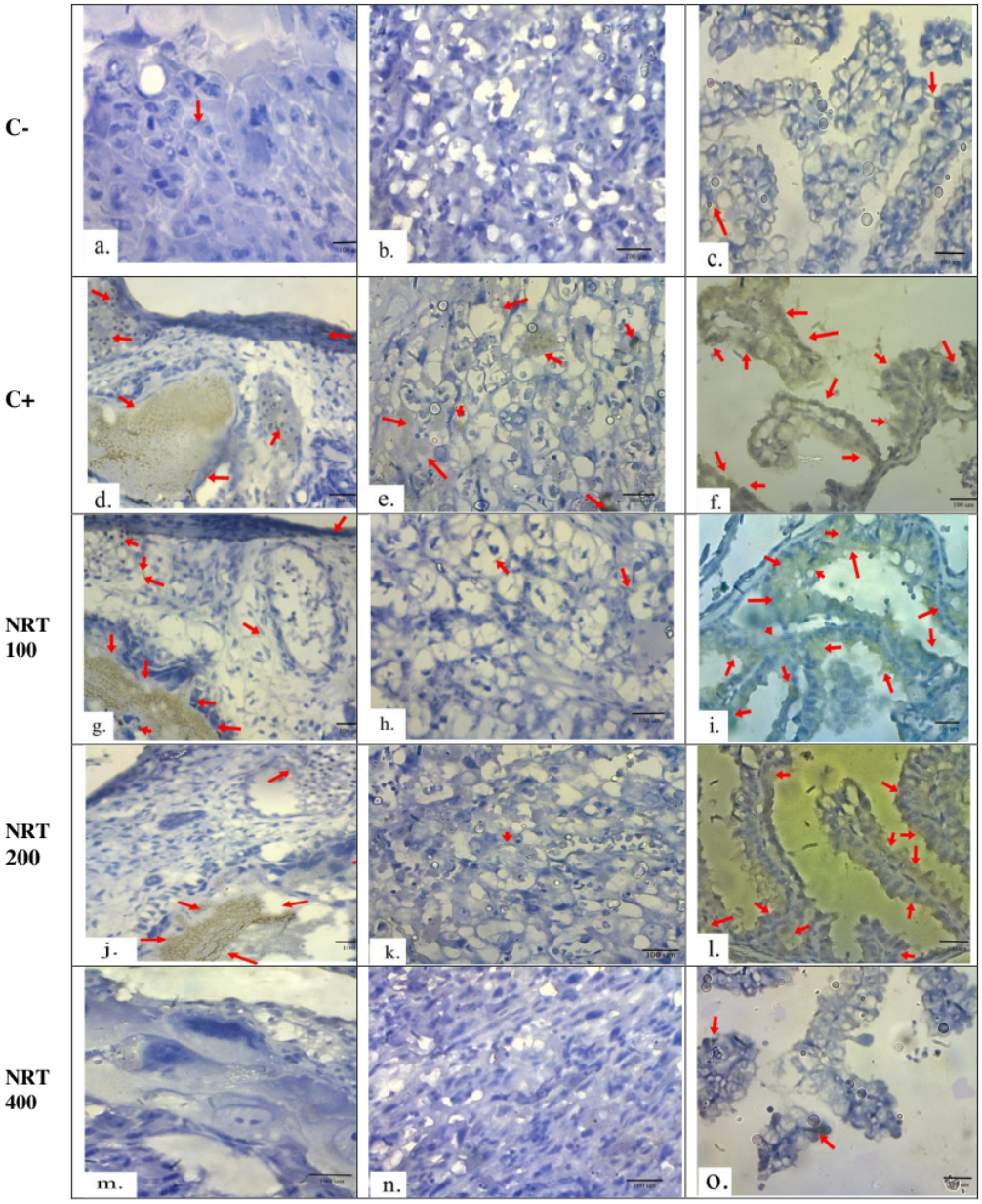

Fig.4. Fas expression in hypertensive rat placenta histology. C-: Untreated, C+: Hypertension rat, NRT100: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 100mg/kgBW, NRT200: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 200mg/kgBW, NRT400: Hypertension rat given by Nano herbal *Rhodomyrtus tomentosa* 400mg/kgBW. (40x).

4. Conclusion

To summarize, the findings show that hypertension causes a loss in placental efficiency in rats, as well as a drop in HSP cause of hypoxia, and an increase in Fas expression, an apoptotic pathway that results in a reduction in fetal birth weight. In hypertensive pregnancies, NRT (haramonting) treatment improves placental function and birth weight.

Running title
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Conflict of interest
The authors declare that they have no conflict of interest

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