

# PROTECTION OF ESTROGEN IN PORTAL HYPERTENSION GASTROPATHY – an experimental model

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**ABSTRACT** – *Context* - Portal hypertension is a complication secondary to cirrhosis that is characterized by increased blood flow and/or vascular resistance in the portal system, causing the appearance of a hyperdynamic collateral circulation. Partial portal vein ligation is an experimental model used in rats to study the pathophysiological mechanisms involved in pre-hepatic portal hypertension. Estrogen E2 is an antioxidant molecule with various physiological actions. *Objectives* - To evaluate the antioxidant activity of endogenous estrogen in an experimental model of partial portal vein ligation by comparing intact with castrated rats. *Methods* - Twenty Wistar rats, weighing on average 250 g were used and divided into four groups: sham-operated (SO); intact (I) with partial portal vein ligation (I + PPVL), castrated (C) and castrated with partial ligation of the vein (C + PPVL). Day 1: castration or sham-operation; day 7, PPVL surgery; on day 15 post-PPVL, portal pressure in the mesenteric vein of rats was measured on polygraph Letica. Lipid peroxidation in the stomach was assessed using the technique of thiobarbituric acid reactive substances and activity of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. Statistical analysis was done with ANOVA - Student-Newman-Keuls (mean ± SE), and  $P < 0.05$  was considered as significant. *Results* - Portal pressure was significantly increased in C + PPVL as compared to the other groups. There was no significant difference in the group of intact rats. TBARS showed significant damage in C and C + PPVL in relation to others. Antioxidant enzymes were significantly increased in the castrated rats with subsequent PPVL as compared to the other groups. *Conclusion* - We suggest that estrogen E2 plays a protective role in intact compared with castrated rats because it presents hydrophenolic radicals in its molecule, thus acting as an antioxidant in this experimental model.

**HEADINGS** - Stomach diseases. Hypertension, portal. Estrogens. Rats.

## INTRODUCTION

Portal hypertension (PH) is a complication of cirrhosis characterized by dilatation of the mesenteric vessels with an increase in portal pressure (PP) and development of collateral circulation which diverts blood from the portal to the systemic circulation, bypassing the liver and leading to the formation of gastroesophageal varices and dilatation of the gastric submucosa, a situation that characterizes portal hypertensive gastropathy (PHG)<sup>(6, 23)</sup>.

PH results from an obstruction of the blood flow in vessels caused by different disorders that may occur in pre-, intra- or post-hepatic anatomical locations, respectively, portal thrombosis, cirrhosis and Budd-Chiari syndrome<sup>(10, 11)</sup>.

The attempt to decompress the portal system causes the communication between the portal and systemic circulation vessels to become dilated and tortuous, and one of the most common locations is the submucosa of the stomach and esophagus.

The largest component of gastroesophageal varices is formed as a result of anastomosis between the left gastric vein, part of the portal system, and the azygos vein, which drains into the superior vena cava. There may be anastomosis between perisplenic vessels (components of the portal system) and short gastric veins draining into the superior vena cava, constituting the main source for varicose veins of the submucosal plexus of the stomach and for PHG<sup>(22)</sup>.

PHG is characterized by vascular changes occurring predominantly in the gastric submucosa, including red spots or “gastric petechiae”, marked vascular ectasia, and absence of inflammation<sup>(23, 30)</sup>.

The changes in blood flow cause the release of vasoactive substances produced by the endothelium as vasodilators (prostacyclin and nitric oxide) and vasoconstrictors (endothelins and prostanoids) that act in paracrine fashion in smooth muscles<sup>(22)</sup>.

The model for induction of portal hypertension in rats was described by Sikuler et al.<sup>(34)</sup>, in which

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partial portal vein ligation (PPVL) is performed, thus increasing the portal venous flow without compromising the liver function, as it is a pre-hepatic model. This model allows to evaluate different stages of PH, the interaction between flow and resistance of the portal system, the relationship between the development of hyperdynamic circulatory state and the elevation of the portal venous flow, and to assess the actions of different drugs that alter the degree of PP<sup>(33)</sup>.

Hemodynamic changes involved in PH lead to increased reactive oxygen species (ROS) in the gastric mucosa, which trigger oxidative stress (OS)<sup>(26)</sup>. Both the hemodynamic changes and the OS affect men and women differently, as described in different randomized studies indicating that the incidence of coronary heart disease is lower in pre-menopausal women than in men the same age, and pointing to an increase of cardiovascular diseases in menopause<sup>(32)</sup>. According to Borrás et al.<sup>(4)</sup> the mitochondria are responsible for differences in longevity between genders, concluding that mitochondrial oxidant production is higher in males than in females. In another study, Borrás et al.<sup>(5)</sup> found that these effects are due to the action of estrogen (E2), which self-regulates the expression of nuclear genes of antioxidant enzymes acting directly on the mitochondria. Thus, E2, with its action as a hormone and its antioxidant activity, contributes to the protective effect in females.

Ovariectomy (castration) is the surgical removal of the ovaries to induce menopause (absence of estrogen). Thus, after the process of castration there is a decrease in serum E2 as well as a reduction of the weight of uterine horns<sup>(36)</sup>.

In cells, E2 can inhibit apoptosis of endothelial cells and promotes angiogenic activity *in vitro*<sup>(15)</sup>. Estrogens are thus considered vasoprotective agents<sup>(20, 41)</sup>. They act on different intracellular mechanisms due to their antioxidant activity by having a hydrophenolic group in their molecule, which grants them action as scavenger of free radicals, decreasing the formation of ROS<sup>(28)</sup>.

Given that PH leads to the development of a hyperdynamic circulation and oxidative stress, the aim of this study was to investigate the action of E2 in the stomach of female Wistar rats subjected to castration and PPVL.

## METHODS

### Animals

Twenty female Wistar rats weighing 250 g were used. The animals were obtained from the State Foundation for Production and Research in Health (FEPPS), Porto Alegre, RS, Brazil. They were kept at the vivarium of the Lutheran University of Brazil in plastic boxes measuring 47 cm x 34 cm x 18 cm lined with wood chips, in a 12-hours dark/light cycle (light from 7 a.m. to 7 p.m.) at a temperature of 22°C ± 4°C. They were fed 16 g per animal/day on rat chow (Purina-Nutripal, Porto Alegre, RS, Brazil) and had water *ad libitum*.

This experiment complied with the norms of the ethics committee on animal research of the Lutheran University of Brazil, Canoas, RS, Brazil. (Protocol # 55-2010).

### Experimental design

The rats were divided into four groups (n = 5): SO (sham-operated), which underwent simulation of both ovariectomy and partial portal vein ligation; intact (I) + PPVL, which were submitted to PPVL but sham ovariectomy; C (castrated), submitted to ovariectomy but sham PPVL; and C + PPVL, submitted to both ovariectomy and PPVL.

### Ovariectomy

The procedures for castration (groups C and C + PPVL) and simulation (groups SO and I + PPVL) were performed on the 1st day of the experiment. The rats were anesthetized with ketamine (ketamine chloride, Parke-Davis, São Paulo, SP, Brazil) in a dose of 100 mg/kg and xylazine (Xylazine chloride - LAB) 50 mg/kg IP and surgery was performed bilaterally according to the model of Baker et al.<sup>(1)</sup>.

### Partial portal hypertension induction

The animals were anesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg) IP. After a medium incision in the abdomen, the bowels were gently withdrawn on humidified gauze with saline and the portal vein was isolated. A 20 g needle was placed into the portal vein and both were tied up using a 3.0 silk yarn, the needle being gently withdrawn after ligation. Subsequently, we ensured that portal vein thrombosis did not occur by manipulating the spot<sup>(34)</sup>.

The SO and C groups underwent the same procedures but their portal veins was not submitted to partial portal vein ligation.

### Portal pressure measurement

Portal pressure was measured in mm Hg on polygraph 2006 (Leticia Scientific Instruments, Barcelona, Spain) by cannulation of the mesenteric vein with a catheter.

### Death of animals

The animals were killed after the recording of portal pressure by exsanguination under deep anesthesia. The stomach was removed and weighed, a small fragment was separated and immersed in buffered formalin for histological analysis, and the remainder was homogenized and frozen at -80°C for subsequent biochemical evaluations.

The uterine horn of rats was removed and immediately weighed (wet weight), and then placed in an oven for 24 hours for subsequent measurement of dry weight.

### Stomach homogenates

The stomachs were cut with scissors and weighed. Nine millilitres of phosphate buffer (140 mM KCL, 20 mM phosphate, pH 7.4) per tissue gram was added, and the tissue was homogenized in an Ultra Turrax (IKA-WERK) for 40 seconds at 4°C. Next, it was centrifuged for

10 minutes at 4,000 rpm (2150,4 x g) (SORVALL RC-5B Refrigerated Superspeed Centrifuge). The supernatant was pipetted into Eppendorf flasks, and the precipitate was discarded. The samples were stored again at -80°C for posterior analyses<sup>(21)</sup>.

**Protein**

We used the Bradford<sup>(8)</sup> method to quantify protein, with bovine albumin as the standard (SIGMA<sup>(8)</sup>). The samples were measured spectrophotometrically at 595 nm, and values expressed in mg/mL were used to calculate values of TBARS (thiobarbituric acid-reactive substances) and antioxidant enzymes.

**Stomach lipoperoxidation**

The amount of aldehydes generated by lipid peroxidation is measured by the TBARS method, which measures the amount of substances reacting with thiobarbituric acid. The samples were incubated at 100°C for 30 minutes after addition of 500 µL of 0.37% thiobarbituric acid in 15% trichloroacetic acid and centrifuged at 3,000 rpm (1612.8 x g) for 10 minutes at 4°C. Absorbance was determined spectrophotometrically at 535 nm<sup>(9)</sup>.

**Antioxidants enzyme analyses**

The analysis of superoxide dismutase (SOD) is based on the inhibition of the reaction of the superoxide radical with adrenaline, values expressed in U/mg prot<sup>(24)</sup>. The analysis of catalase (CAT) activity is based on measuring the decrease in hydrogen peroxide, values expressed in pmol/mg prot<sup>(7,37)</sup>. The activity of glutathione peroxidase (GPx) is based on the consumption of NADPH in the reduction of oxidized glutathione<sup>(39)</sup>, values expressed in mmoles/min/mg prot<sup>(14)</sup>.

**Histology**

For histological evaluation, part of the stomach was preserved in 10% formalin for 24 hours, embedded in paraffin and cut into 3 µm sections with a microtome. The sections were stained with hematoxylin-eosin (100x).

**Statistical analysis**

All data are presented as means ± SE. Statistical significance was calculated using Graphpad Instat, version 3.0 for Windows. We used variance analysis (ANOVA) and Student-Newman-Keuls for multiple analysis, adopting a significant level of 5% ( $P<0.05$ ).

**RESULTS**

**Efficacy of castration**

To evaluate the efficacy of surgical castration, the difference between wet and dry weight of uterine horns was calculated. The uterine horns of groups C and C + PPVL had significantly lower values ( $P<0.05$ ) than those of groups I and SO + PPVL for both wet and dry weights (Figure 1).

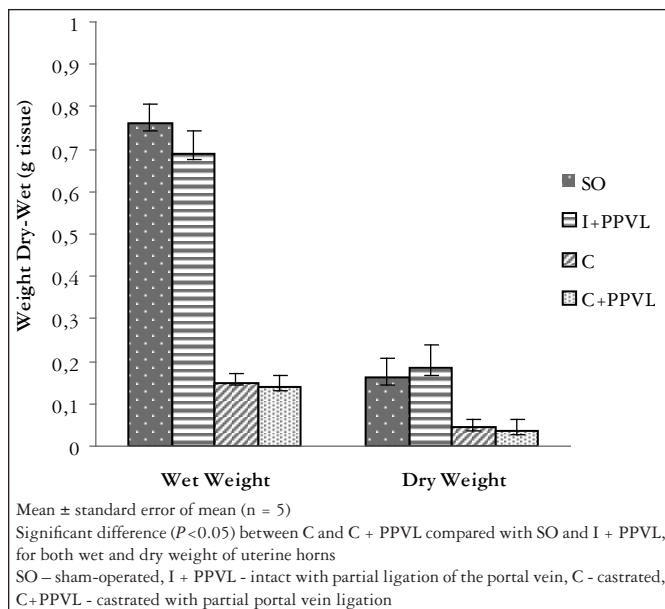


FIGURE 1. Wet and dry weights of uterine horns of rats subjected to surgical castration and intact rats

**PP**

The analysis of the results of PP in the different experimental groups showed a significant increase in C + PPVL as compared to the other groups ( $P<0.05$ ). It should be highlighted that there was no increase in PP in I + PPVL compared with SO and C, despite PPVL (Figure 2).

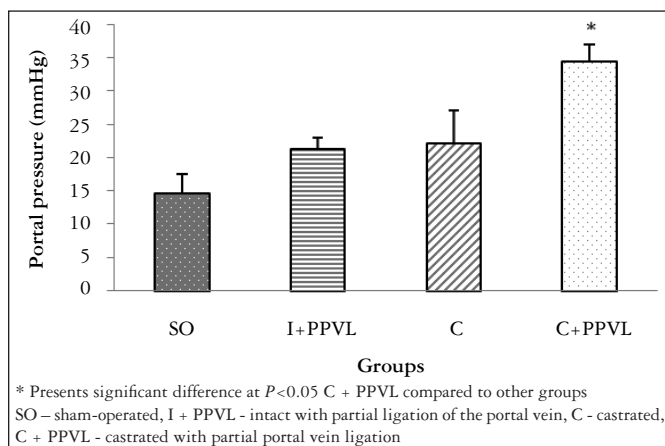


FIGURE 2. Average values of portal pressure (mm Hg) across the different groups. Mean ± standard error of mean (n = 5)

**Lipoperoxidation (LPO)**

Regarding LPO values obtained by TBARS in homogenates of stomach, there was a significant increase ( $P<0.05$ ) of LPO in C and C + PPVL in relation to I and SO + PPVL (Figure 3), showing that castration led to an increase in lipid peroxidation. SO and I + PPVL, in which the ovaries and therefore circulating estrogen were maintained, showed no significant difference concerning LPO in stomach.

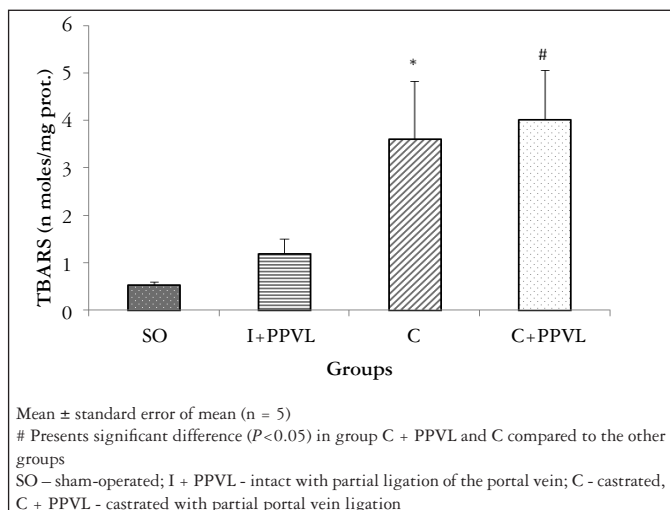


FIGURE 3. Average TBARS values in different groups

### Antioxidant enzyme activity

The activities of antioxidant enzymes SOD, CAT and GPx were evaluated in homogenized stomach. The data are shown in Table 1. The antioxidant enzymes did not show any significant differences in groups I and SO + PPVL, while groups C and C PPVL showed a different profile.

TABLE 1. Values of SOD, CAT and GPx activities

	SO	I+PPVL	C	C+PPVL
SOD (USOD/mg prot)	26.33 ± 5	24.17 ± 1,6	80.73 ± 8.5*	112.64 ± 9.8#
CAT (pmoles/mg de prot)	0.14 ± 0.02	0.16 ± 0.01	0.24 ± 0.005*	0.35 ± 0.06#
GPx (nmoles/mini/mg prot)	0.67 ± 0.28	0.51 ± 0.18	2.9 ± 0.05*	0.57 ± 0.02

# \*Significant increase ( $P < 0.05$ ) of SOD and CAT in groups C and C + PPVL compared to the other groups

\* Significant increase ( $P < 0.05$ ) in GPx compared to the other groups

Mean ± standard error of mean (n = 5). SO – sham-operated, I + PPVL - intact with partial ligation of the portal vein, C - castrated, C + PPVL - castrated with partial portal vein ligation

Group C showed a significant increase ( $P < 0.05$ ) of SOD and CAT activities in relation to groups I and SO + PPVL, while group C + PPVL showed a significant increase ( $P < 0.05$ ) of these enzymatic activities in relation to all groups.

Concerning GPx activity, there was a significant increase ( $P < 0.05$ ) in group C compared to the other groups, and group C + PPVL showed a significantly decreased GPx activity compared to group C.

### Histology

The histological analysis of stomach tissues showed edema, congestion, and vessel proliferation in the gastric mucosa of animals of groups I + PPVL and C + PPVL (Figure 4 (3 and 4)), in contrast to groups SO and C, which were not submitted to PPVL (Figure 4 (1 and 2)).

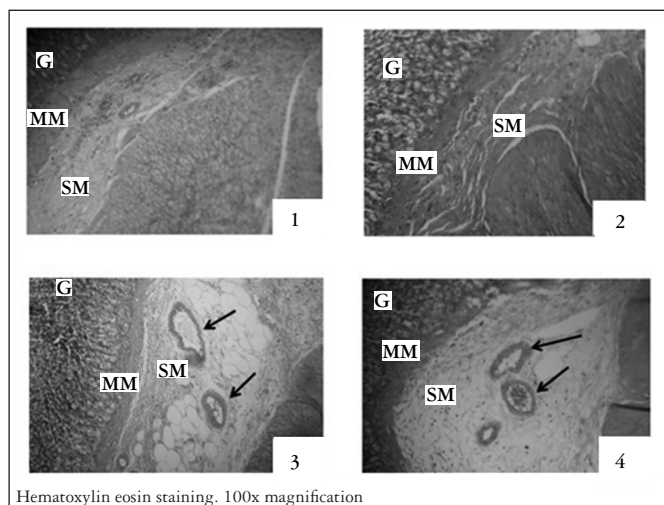


FIGURE 4. Micrograph of gastric tissue of an animal in SO (1), animal in group C (2), animal in group I + PPVL (3), and animal in group C + PPVL (4) G: glandular tissue; MM: muscularis mucosae; SM: submucosa Edema, congestion and proliferation of blood vessels can be seen in images C and D. The arrows in images C and D show the dilated vessels in the animals of groups I + PPVL and C + PPVL, respectively

### DISCUSSION

Castration (ovariectomy) induces surgical “menopause”, and 7 days after surgery ovariectomized animals have no detectable levels of estrogen<sup>(27)</sup>. Torrezan et al.<sup>(36)</sup> demonstrated that ovariectomy decreases the weight of uterine horns, a fact that was confirmed in our study, comparing the efficacy of surgery as well as evidencing the absence of circulating estrogen (Figure 1).

Borekci et al.<sup>(18)</sup> demonstrated the protection of estrogen in the gastric mucosa, as other authors have demonstrated the greatest protection in the cardiovascular system in women of reproductive age compared with men the same age, for their hormonal and antioxidant activity<sup>(3, 12, 13, 17, 27)</sup>.

Intact or castrated female rats were subjected to PPVL (pre-hepatic model) and then analyzed regarding the influence of castration (absence of estrogen) in PH-related changes, mainly to PHG.

A previous work by our group<sup>(26)</sup> and other authors<sup>(6, 34, 38, 40)</sup> showed that on day 15 post-PPVL there is a significant increase in PP in all animals. In the present study with female rats subjected to PPVL, the intact rats showed no significant increase in PP, but in the castrated rats PP was significantly increased ( $P < 0.05$ ) (Figure 2). Intact rats with PPVL and castrated rats without PPVL were found to have the same profile of greater portal pressure as compared to SO animals, a finding that was not significant but may be considered as an effect of the absence of estrogen by castration. The absence of estrogen and PPVL potentiate the actions causing PP increase. We note that in intact rats, therefore with estrogen, PPVL appears not to cause the usual increase in PP, which seems to indicate a degree of protection by estrogen preventing that significant increase. This protection could possibly be related

to the action of E2 on the endothelial function by modulating the generation of NO, thereby contributing to vasodilation as shown by a number of authors<sup>(16, 19, 25, 41)</sup>.

Oxidative stress measured by lipid peroxidation through TBARS is increased in rats subjected to PPVL. In Figure 3 one sees that intact rats showed no significant increase in TBARS, which were similar to those of SO. In castrated rats with and without PPVL there was a marked increase in TBARS ( $P < 0.05$ ). The absence of estrogen shows a significant increase in TBARS and thus in LPO, with more cellular damage.

Estrogen, seemingly protecting against LPO, could be exerting its potential antioxidant preventing oxidative damage and oxidative stress, as previously demonstrated<sup>(2, 5, 18, 28)</sup>, through its hydrophenolic radicals<sup>(27)</sup>.

Regarding antioxidant enzymes, SOD and CAT showed similar activity across the groups. In SO and I + PPVL there was no significant difference ( $P < 0.05$ ) of SOD and CAT, as we found no difference in the LPO (Figure 3). Since in the I + PPVL the ovaries were kept, we suggest that E2 is conferring this protective action.

Römer et al.<sup>(31)</sup> reported that several estrogen metabolites inhibit the production of superoxide radical anion in vitro and influence in the iron redox cycle, leading to less free radical formation. Likewise, Borrás et al.<sup>(4)</sup> found that mitochondria of females have a higher expression of antioxidant enzymes than males. In our study, we observed in group I + PPVL that estrogen granted protection, as there was an increase in LPO in this group, explaining the SOD values found.

In groups C and C + PPVL a significant increase in the activity of these enzymes was observed as compared to the other groups (Table 1). We suggest that this increase may have occurred to compensate for the absence of circulating E2, promoted by castration, but even then it was not effective in reducing LPO as shown in Table 1. Other authors have

reported that castration increases LPO by increased production of hydrogen peroxide<sup>(35)</sup>, corroborating our findings, as well as studies such as Ozgonul et al.<sup>(29)</sup>, in which castrated female Sprague-Dawley rats have increased CAT activity in liver and estrogen replacement reverses this effect, returning to the values of the control animals.

Hernández et al.<sup>(17)</sup> demonstrated that castration caused a decrease in thiol groups (-SH) and total antioxidant capacity in plasma as well as an increase in plasma lipoperoxides, reflecting an imbalance in favor of oxidative processes.

In our experimental model as well as in Hernández et al.<sup>(17)</sup>, castration triggered a significant increase in LPO (Figure 3). We suggest that castration may have reduced the thiol groups, consistent with the significant decrease in GPx activity in C + PPVL, favoring the damage, which was evidenced by the increased LPO in this group as compared to the others. In contrast, group C showed a significant increase in GPx as compared to the others, in an attempt to compensate for the lack of circulating estrogen.

Analyzing the micrographs of the different experimental groups, we noticed that in groups C and SO there was no vasodilation (Figure 4 (1 and 2)) while in groups I + PPVL and C + PPVL there was vasodilation, congestion and edema in the sub-mucosa (Figure 4 (3 and 4)), characteristic of this model that leads to PHG<sup>(22, 23, 26)</sup>. Although the group I+PPVL did not show changes in PP and LPO, histology already showed damages resulting from PPVL.

## CONCLUSION

We suggest that E2, by presenting hydrophenolic radicals in its molecule and acting as a scavenger of free radicals, may have protected the intact rats from the hemodynamic changes and LPO, in the comparison of castrated and intact Wistar rats in this experimental model.

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**RESUMO – Contexto** - A hipertensão portal é uma complicação secundária à cirrose que tem como característica aumento do fluxo sanguíneo e/ou resistência vascular no sistema porta, causando o surgimento de uma circulação colateral hiperdinâmica. A ligadura parcial de veia porta é o modelo experimental utilizado em ratos para estudar os mecanismos fisiopatológicos envolvidos na hipertensão portal pré-hepática. O estrogênio E2 é uma molécula antioxidante com diferentes ações fisiológicas. **Objetivos** - Verificar a ação antioxidante do estrogênio endógeno em modelo experimental de ligadura parcial de veia porta comparando ratas intactas com ratas castradas. **Métodos** - Foram utilizadas 20 ratas Wistar, pesando em média 250 g, divididas em quatro grupos: “sham-operated” (SO); intactas com ligadura parcial da veia porta (I + LPVP); castradas (C) e castradas com ligadura parcial da veia porta (C + LPVP). No 1º dia: castração ou “sham-operated”; no 7º dia cirurgia de LPVP; no 15º dia após a LPVP, foi verificada a pressão portal na veia mesentérica das ratas, no polígrafo Letica. A lipoperoxidação no estômago foi avaliada através da técnica das substâncias reativas ao ácido tiobarbitúrico e a atividade das enzimas antioxidantes superóxido dismutase, catalase e glutatona peroxidase. A análise estatística utilizada foi ANOVA - Student-Newmann-Keuls, (Média ± EP) e foi considerado significativo para  $P < 0.05$ . **Resultados** - A pressão portal mostrou aumento significativo no grupo C + LPVP em relação aos demais, não houve diferença significativa no grupo das ratas intactas. O TBARS mostrou dano estatisticamente significativo no grupo C e C + LPVP em relação aos demais. Quanto às enzimas antioxidantes, as ratas castradas e com posterior ligadura parcial de veia porta tiveram aumento significativo em relação às demais. **Conclusão** – Sugere-se que o estrogênio E2, por apresentar radicais hidrofênicos em sua molécula, desempenha um papel protetor nas ratas intactas em comparação com as castradas, agindo assim, como antioxidante, neste modelo experimental.

**DESCRIPTORES** - Gastropatias. Hipertensão, portal. Estrogênio. Ratos.

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