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Indomethacin Reduces Splenic Red Pulp Macrophage Populations in Female New Zealand White Rabbits

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East Tennessee State University

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A Dissertation

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of the Requirements for the Degree

Doctor of Philosophy in Biomedical Science

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by

Thane S. Thurmond

May 1995

APPROVAL

This to certify that the Graduate Committee of

Thane S. Thurmond

met on the

3rd day of April, 1995

The committee read and examined his thesis, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Graduate Studies in partial fulfillment of the requirements for the dgree Doctor of Philosophy in Biomedical Science.

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ABSTRACT

Indomethacin Reduces Splenic Red Pulp Macrophage Populations in Female New Zealand White Rabbits.

by

Thane S. Thurmond

In an effort to elucidate the mechanism by which indomethacin (IN) attenuates the stimulatory effect of estradiol (E_2) on rabbit splenic red pulp macrophages (RPM), thirty-nine female New Zealand White rabbits were divided into 10 groups: ovariectomized (OVX), OVX/IN at 0.1 and 5.0 mg/kg body weight (bw)/day; sham OVX (SOVX), SOVX/IN at 0.1 and 5.0 mg/kg bw/day; OVX/25 mg E_2, OVX/25 mg E_2/IN at 0.1 and 5.0 mg/kg bw/day; intact Control. Quantitative changes in RPM population in response to the treatments were measured using a 0 to 4 histologic grading scale. Estradiol treatment resulted in increased RPM grade when compared to the OVX non-E_2 groups. Indomethacin addition decreased mean RPM grade in the SOVX/IN 5.0 group when compared to its E_2 control group. Indomethacin administration had no significant effect on levels of PGE_2 in the spleen, blood or urine ($p>.05$). Hematocrits were reduced in both OVX and OVX/E_2 groups and this decrease was exacerbated by the high IN dose. The results from this study suggest that the effect of IN on E_2-induced RPM activation may be mediated through a non-prostaglandin pathway. The observed hematocrit changes are possibly the result of direct action of IN and E_2 on erythrocytes.

To further investigate whether a direct interaction of IN and E_2 with rabbit erythrocytes may be responsible for the decreases in hematocrit observed *in vivo*, an *in vitro* study was conducted to determine the effect of these drugs on erythrocyte fragility characteristics. Two ml aliquots of treated New Zealand White rabbit whole blood were assayed as; Control, IN (9.6 µg/ml), E_2 (500 pg/ml) and IN plus E_2, for changes in erythrocyte fragility. Osmotic (OF) and mechanical (MF) fragility were evaluated under approximate physiological conditions by measurement of hemoglobin release at 545 nm. Blood samples at 39.5°C were assayed immediately after drug addition (initial) and again 4 hours after incubation (final). Eight replicates of each experiment were run. Results of the OF assays showed a significant increase ($p<.05$) in mean 50% hemolysis point between IN (final) and IN plus E_2 (final) when compared to their mean initial values and to the mean final Control value. The OF hemolysis dispersion was increased by IN and IN plus E_2 treatment when final values were compared to initial values. The mean final values for MF increased with IN, E_2 and IN plus E_2 treatment versus the mean final Control value ($p<.05$). While the increase in MF from IN was greater than that from E_2, the MF from the combination (IN plus E_2) was not greater than from IN alone ($p>.05$). The IN-induced increases in both OF and MF indicate a difference in degree of interaction with the erythrocyte from that of E_2, which only affected MF and whose effect was not additive or synergistic with that of IN.

The *in vitro* experimental results demonstrate that the increased fragility produced by IN and E_2 on rabbit erythrocytes may account for the observed *in vivo* reduction in hematocrit. Increased erythrocyte fragility would also lead to their accelerated clearance from the circulation by splenic RPM and subsequent increases in activity of these macrophages. This elevation in splenic RPM population may also be enhanced by direct E_2 stimulation of macrophage proliferation.

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Chapter 1

INTRODUCTION

Previous Research

Coogan *et al.* (1981) found that E_2, administered over a period of 10 to 12 months, produced multicentric, glycogen-rich, reticuloendothelial tumors in the spleen and renal medulla of sexually mature, female, New Zealand White (NZW) rabbits. Subsequent work demonstrated that similar changes in the splenic reticuloendothelial system could be induced in as little as three weeks by subcutaneous implantation of 25 mg sustained-release E_2 pellets in ovariectomized NZW rabbits (Zollars *et al.*, 1985). The splenic alterations observed in this study consisted of a splenomegaly characterized by reticuloendothelial cell (REC) hypertrophy and hyperplasia.

The exact nature of E_2's effect on the splenic reticuloendothelial and renal medullary cells is not known. However, because both cell types elaborate large amounts of the arachidonate metabolite prostaglandin E_2 (PGE_2) (Stenson and Parker, 1980; Lee *et al.*, 1967) a study was undertaken in our laboratory to determine if PGE_2 may play a role. This investigation was designed to evaluate PGE_2 involvement by co-administration of a prostaglandin pathway cyclooxygenase blocker along with E_2. The cyclooxygenase blocker chosen was indomethacin (IN) which is considered to be one of the more potent of the non-steroidal anti-inflammatory drugs (Insel, 1990). Subcutaneously-implanted, sustained-release IN pellets at dose levels of 1.0, 10.0 and 13.0 mg/kg body weight (bw)/day were administered, along with 25 mg sustained-release E_2 pellets, to sexually mature, ovariectomized, NZW rabbits for a period of three weeks. At

the end of the study sections of spleen from all groups were examined histologically and the two larger IN doses were noted to be effective in reducing the extent of the REC hypertrophy and hyperplasia when compared to the ovariectomized group receiving E_2 alone and the group receiving the low dose of IN (data unpublished).

Based upon the results of this preliminary study, an *in vivo*, 3-way crossover study utilizing 3 treatment regimens was undertaken to further investigate E_2 supplementation-induced splenomegaly and gain insight into IN's actions on the splenic changes. A set of *in vitro* experiments was also conducted which addressed the effects of these two compounds on the fragility characteristics of rabbit red blood cells.

Mononuclear Phagocyte System

The mononuclear phagocyte system consists of promonocytes, peripheral blood monocytes, monoblasts in the bone marrow and tissue macrophages. The macrophage is the primary differentiated cell of this system and is found throughout the body in many structural and functional forms. The first use of the term "macrophage" was by Elie Metchnikoff (Karnovsky, 1981) to describe large mononuclear cells he had observed in tissue sections. Aschoff (1924) included the macrophages under the broad cell classification of the "reticuloendothelial system" (RES). However, because the RES classification contains some cells of a non-macrophage lineage (i.e., endothelial cells, fibroblasts and reticular cells) in 1969 the term mononuclear phagocyte system was adopted to describe monocyte-derived phagocytic cells with common *in vivo* functional characteristics (van Furth *et al.*, 1972). Because of this change in convention the term monocytic-macrophage rather than reticuloendothelial cell will be used in this publication to refer to those phagocytic cells. The monocytic macrophages of interest in these studies are those that reside in the red pulp of the rabbit spleen (RPM).

Estrogens and the Immune Response

General

Estrogens are naturally occurring hormones or synthetic steroidal and non-steroidal compounds that exhibit estrogenic activity. The naturally occurring estrogens have a basic cyclopentanoperhydrophenanthrene steroid ring backbone, with an unsaturated A ring, a C 3 position phenolic hydroxyl group, a methyl group at the C 13 position and a hydroxyl or ketone group at the C 17 position. The natural steroidal hormones consist of estradiol, estriol, estrone, equilenin and equilin; of these, estradiol (E_2) has the greatest estrogenic activity. Estrogens, along with their functions in normal systemic growth and maturation and development of secondary sex characteristics, have also been shown to have a regulatory effect on the immune system in humans and in animals.

Effects of Estrogen on the Thymus, T, B and NK Lymphocytes.

Thymic Effects. Early studies have suggested that the thymus is the major target organ through which estrogens and sex hormones generally exert their immunomodulatory influence. Sobhon and Jirasattham (1974) observed that E_2 benzoate administered to ovariectomized rats resulted in a significant decrease in thymic weight when compared to ovariectomized and sham ovariectomized control animals. In the same study, the authors reported that ovariectomy in immature rats resulted in a delay in thymic involution. In a morphometric study of thymuses from rats receiving E_2 benzoate neonatally, Leceta *et al.* (1988) observed thymic cortical hypoplastic changes characterized by decreases in the frequency of large lymphoid cells in that region. Screpanti *et al.* (1989), in assessing the lymphocyte subpopulations involved in the

estrogen-induced thymic changes, concluded that short-term administration of 17β-estradiol produced a decrease in CD4+/CD8+ (DP) subsets and an increase in subsets CD4+/CD8- (SP), CD4-/CD8- (DN) and, to a lesser extent, CD4-/CD8+, as well as an increase in the proportion of beta TCR+/epsilon CD3+ and IL-2R+ cells. After long-term E_2 administration, they observed a decrease in the effect on DN/IL-2R+ cells.

Differences in thymic response have been observed when E_2 was administered cyclically versus chronic administration. In a study by Erbach and Bahr (1988) the thymuses of ovariectomized rats receiving E_2 on a four-day cycle showed none of the atrophic changes seen in the ovariectomized rats receiving implanted, constant release estradiol pellets.

Effects on T, B and NK Lymphocytes. Research on the effect of estrogens on the immune system has shown that in general estrogenic substances depress T-cell mediated responses and NK cell activity while stimulating the B-cell humoral response (Ahmed *et al.*, 1985). Several researchers have reported that T cells exposed to sex hormones in culture were less responsive to the T-cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) (Mendelson *et al.*, 1977; Clemens *et al.*, 1979; Wyle and Kent, 1977). Bellini and his co-investigators (1990) showed that PHA-stimulated human lymphocytes pretreated with E_2 produce diminished concentrations of cytosolic free calcium in comparison to non-E_2 treated control cells. Clerici *et al.* (1991) reported that treatment of human peripheral blood monocytes (PBMC) with E_2 produced enhanced immune response to sheep red blood cells (SRBC) by inhibiting CD8+ T lymphocytes (suppressor T cells). Further evidence of estrogen's effect on suppressor T-cells was provided by Ho *et al.* (1991) who demonstrated that patients with estrogen deficiency

due to gonadal dysgenesis or hypothalamic-pituitary failure had a decreased CD4(helper T-cell):CD8 ratio and increased numbers of CD8+ cells compared to normal control groups.

Work by Erbach and Bahr (1991) in assessing the effect of E_2 on the B-cell humoral response in ovariectomized Lewis rats found that in order for estradiol humoral enhancement to occur an intact thymus is required. They also observed that a constitutive thymic factor found in thymosin fraction 5 exerts a permissive influence on E_2-induced humoral immune enhancement outside the thymus. Evagelatou and Farrant (1994) noted that thymus derived T-cells were necessary for E_2 action on the differentiation of B-cells in cultures of human tonsillar lymphocytes. Sthoeger *et al.* (1988) demonstrated E_2 enhanced pokeweed mitogen (PWM)-induced generation of plaque forming cells in cultures of human peripheral blood mononuclear. Similar results for E_2-potentiation of IgG and IgA production by PWM-stimulated B-cells from multiple sclerosis patients were reported by Kalman *et al.* (1989).

Seaman and Talal (1980) in their work on estrogen-related immune system changes, reported that NK activity was markedly reduced in E_2-treated B/W mice. Other authors have reported similar NK cell activity changes in E_2-treated TA3 mice of both sexes (Hou and Zheng, 1988) and in women suffering from endometriosis (Garzetti *et al.*, 1993). Screpanti *et al.* (1987) reported that the E_2-induced NK cell effects were time dependent, noting that E_2 administration in mice for up to 30 days actually stimulated NK cell activity while longer administration depressed activity.

Effects of Estrogen on Cells of the Mononuclear Phagocyte System

Estrogenic effects on the cells that compose the mononuclear phagocyte system have been well documented. Fluhmann (1928; 1932) first reported a decreased presence of vitally stained macrophages in the endometrium of ovariectomized rabbits. Nicol (1932; 1935) demonstrated comparable changes in the reticuloendothelial system of the guinea pig uterine horn endometrium. Both researchers also demonstrated that normal macrophage levels could be restored by administration of natural estrogens. Subsequent work by Nicol's group and others confirmed that both natural and synthetic estrogens produced similar effects (Heller *et al.*, 1957; Nicol and Bilbey, 1960; Nicol *et al.*, 1966). Estrogens also stimulate resident mononuclear phagocytes, inducing Kupffer cell division in the liver (Kelly and Dobson, 1971; Boorman *et al.*, 1980; Dean *et al.*, 1984; Durquet-Pereimann *et al.*, 1990) and increasing activity of splenic red pulp phagocytes (Numano *et al.*, 1969; Warr and Sijivic, 1973; Zollars *et al.*, 1985).

Although estrogenic stimulatory effects on mononuclear phagocytes have been well established, little is understood about how these estrogen-related changes are induced. The likelihood of direct estrogen involvement is given credence by the demonstration of estrogen receptors in human peripheral monocytes (Weusten *et al.*, 1986) and in the J111 human monocytic leukemia cell line and rat peritoneal macrophages (Gulshan *et al.*, 1990). Brubaker and Gay (1994) also reported specific, dose and time dependent binding of estradiol to the surface of avian osteoclasts (a subset of the monocyte-macrophage system). This binding resulted in decreased osteoclast acid production and changes in cell shape. Administration of physiological and pharmacological levels of estradiol has been shown to induce the production of interleukin-1 (IL-1) in murine peritoneal macrophages (Barañao *et al.*, 1992; Hu *et al.*, 1988; Flynn, 1986) and in LPS-stimulated splenic macrophages from oophorectomized rats (Sato *et*

al. 1993). Interleukin-1 has been shown to stimulate T-lymphocytes to produce the cytokines interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF), both of which stimulate the production of peripheral monocytes (Cannistra *et al.*, 1987). Fibroblasts and endothelial cells are also sensitive to IL-1 and respond by producing GM-CSF and macrophage colony-stimulating factor (M-CSF) (another potent stimulator of mononuclear phagocyte production) (Bagby *et al.*, 1986). Whether IL-1 is the mediator for the estrogen-induced stimulation of macrophage production is speculative since recent work has demonstrated that the production of individual macrophage-related cytokines may relate to the level of maturation and anatomic location of the macrophage (Friedlander *et al.*, 1994; Rice *et al.*, 1981). Recent studies have also demonstrated that estrogen can directly induce the production of M-CSF by uterine epithelial cells in the cycling mouse (Wood et al., 1992).

Other possible macrophage-related actions of estrogens may correlate to their effect on F_c and complement receptor mediated phagocytosis. Friedman *et al.* (1985) and Schreiber *et al.* (1988) reported that estrogenic compounds enhanced the $F_c\gamma$ receptor-mediated clearance of IgG-coated erythrocytes by splenic macrophages. Barañao *et al.* (1991) showed that peritoneal adherent mononuclear cells from ovariectomized Balb/c mice had decreased functionality of membrane complement C_{3b} receptors and reduced ability to phagocytize *Candida albicans.* Injection of normal levels of estradiol restored phagocytic properties to normal but failed to restore membrane C_{3b} receptors.

Macrophages produce large quantities of the arachidonic acid-derived prostaglandins in response to diverse stimuli, but the amounts of each type produced vary among macrophage populations. Endotoxin, zymosan or microorganism-stimulated peritoneal macrophages produce large amounts of PGE_2, PGI_2 and thromboxane A_2.

Peripheral blood monocytes in contrast, produce only small amounts of PGE_2 and PGI_2, but large amounts of thromboxane (Tripp *et al.*, 1986). The prostaglandins produced by macrophages not only have an effect on vascular permeability but also on the macrophages themselves. Prostaglandin E_2, for example, is one of the most potent inhibitors of interferon γ-induced macrophage activation (Snyder *et al.*, 1982; Taffet and Russell, 1981). The effect of estrogens on macrophage prostaglandin production and consequent activation has been addressed by several investigators. Work by Stratton *et al.* (1986) showed that peripheral monocytes from normal menstruating women produced lower levels of PGE_2 and TxB_2 and had higher levels of phagocytic activity when serum estrogen levels were elevated. Miyagi *et al.* (1993) reported similar results using peripheral monocytes exposed to LPS and E_2 *in vitro*. In their study, however, they reported that while the low dose of E_2 (0.4 ng/ml) depressed PGE_2 production, high levels (20 ng/ml) resulted in increased PGE_2 production. Leslie and Dubey (1994) also noted that LPS stimulation of human peripheral monocytes isolated during the luteal phase of the menstrual cycle had enhanced prostaglandin production when compared with LPS-treated monocytes isolated during the early follicular phase.

The Non-steroidal Anti-inflammatory Drugs

General

The non-steroidal anti-inflammatory drugs (NSAIDs) have as a common property the ability to inhibit the production of the inflammatory prostaglandins. The salicylate-containing NSAIDs have been used for centuries in many cultures to treat a variety of inflammatory conditions including the arthritides. Newer non-salicylate NSAIDs that, in some instances, proved to be more effective in the treatment of arthritis

than the salicylates and had fewer side effects, began to be developed in the late 1960's and early 1970's. The first of these new NSAIDs were indomethacin and phenylbutazone. Indomethacin was developed by chemists at Merck, Sharpe and Dohme based upon the hypothesis that the basic indole structure is similar to that of serotonin, thought to be a major mediator of inflammation. Phenylbutazone, a pyrazolone derivative, was created as result of the efforts of researchers at Geigy in Basel, Switzerland. Since the development of these NSAIDs hundreds more have been tested for their efficacy in the alleviation of the pain and inflammation of the rheumatic diseases; of these drugs, some 30 have been found effective in the treatment of these conditions.

Modes of action in inflammation and the immune response.

One of the primary routes by which NSAIDs produce their effects is by inhibition of the cyclooxygenase component of the prostaglandin synthesis pathway. Blockade of this pathway has been found to play a role in the NSAID modulation of the immune response on several levels although some observed effects have been contradictory (Cortet and Duquesnoy, 1991). Martinez and Coleman (1989) demonstrated that the presence of indomethacin, piroxicam, ibuprofen or aspirin during the first 24 hours of culture of PWM-stimulated (T and B cell mitogen) human peripheral mononuclear cells resulted in a marked decrease in polyclonal IgG synthesis. They noted that the degree of IgG production inhibition was related to the relative abilities of the NSAIDs to inhibit cyclooxygenase. Skoldstam *et al.* (1982a; 1982b) have observed that indomethacin blockade of PGE_2 augments Con A-induced immunosuppression in patients with rheumatoid arthritis, while others have reported an indomethacin-induced depression of the immunosuppressive response in mice in which an autoimmune state had been created (Nicklin and Shand, 1982). Badger *et al.* (1982) reported that indomethacin augmented

the Con A produced suppression of antibody synthesis to sheep red blood cells in Balb/C mice, although they expressed doubt concerning the involvement of prostaglandins in this response.

While cyclooxygenase blockade is the primary mechanism of NSAID action, several drugs within this class have been shown to act via other sites and pathways to suppress the inflammatory process (Rainsford, 1993). Because, as a class, NSAIDs are planar, anionic compounds that partition into lipid environments such as the cell membrane bilayer, it is not surprising that they have been found to inhibit many membrane-associated processes. Indomethacin and the fenamates are unique among NSAIDs in their ability to block phospholipase A_2 (PLA_2) in polymorphonuclear leukocytes (PMN) and in inflamed tissue, thereby inhibiting the production of all prostaglandins, thromboxanes and leukotrienes (Shakir *et al.*, 1985; Franson *et al.*, 1980; Jesse and Franson, 1979). The mechanism of this NSAID blockade is felt to be related to their ability to antagonize the action of Ca^{+2}, which serves as a cofactor for PLA_2 (Northover, 1977). The fenamates have also been found to act directly at prostaglandin receptors, inhibiting the binding of prostaglandins (Rees *et al.* 1988). Indomethacin, piroxicam, ibuprofen (at micromolar concentrations) and aspirin (at millimolar concentrations) inhibit the fMet-Leu-Phe (FMLP)-induced aggregation of PMNs by a non-prostaglandin mediated path (Abramson *et al.* 1984; 1985). Further work by Abramson and his group demonstrated that the neutrophil FMLP aggregation inhibition resulted from uncoupling of postreceptor ligand signaling due to the ability of the NSAIDs to induce changes in membrane fluidity (Abramson *et al.*, 1990) Indomethacin and piroxicam were also found to inhibit the production of superoxide anions by a cell-free membrane preparation of NADPH oxidase (Minta and Williams, 1985);

subsequent work by Yuda et al. (1991) showed that this effect was attributable to direct blockage of this enzyme by the NSAIDs.

NSAID Effect on Mononuclear Phagocytes.

As previously mentioned, the mononuclear phagocytes have the potential to produce large quantities of arachidonic acid metabolites, one of which, PGE_2, acts to suppress further macrophage activation. Blockade of PGE_2 production by the NSAIDs abrogates this inhibitory effect allowing continued macrophage activation. Although blockade of the PGE_2 pathway is considered the primary mechanism of macrophage inactivation, several authors have demonstrated that NSAID administration can have an effect on their activity levels without directly affecting prostaglandin production. Rice *et al.* (1981) in their investigations on liver plasma membranes of Sprague-Dawley rats showed that indomethacin administered *in vivo* directly reduced the binding affinity of PGE_2 without having an effect on PGE_2 levels. Razin *et al.* (1982) demonstrated that *in vitro* administration of indomethacin to mouse peritoneal macrophages produced a significant decrease in membrane binding of IgG-coated sheep erythrocytes and a significant increase in membrane binding of cationized ferritin. Indomethacin administration *in vitro* has also been shown to stabilize mouse peritoneal macrophage lysosomal hydrolases in response to challenge by C-mucopolysaccharide peptidoglycan (Finlay *et al.*, 1975). This effect of indomethacin may be mediated through its antagonism of Ca^{+2}, which has been shown to be involved in the lysosome fusion process in neutrophils (Jaconi *et al.*, 1990) and in the secretion of lysosomal enzymes by human neutrophils induced by FMLP and C5a (Shaw *et al.*, 1982).

Rationale

While well documented, the mechanism or mechanisms of estrogen's stimulatory effect on the monocyte macrophage system is not understood. Questions remain about whether the effect is related to a direct or indirect hormonal interaction with this system, or whether it may be due to a combination of factors. The presence of estradiol receptors in monocytes and other cells of this system make direct estrogen stimulation a possibility. As noted earlier, estrogens also have other effects on the immune system that may indirectly affect monocytic macrophages. This question is especially applicable to splenic monocytic macrophages since the splanchnic organs have been shown to be an important site of first passage uptake of the estrogens (Collins *et al.*, 1970). Another factor to consider is the effect of the estrogens on the circulatory flow characteristics of erythrocytes. Increased erythrocyte membrane rigidity has been reported during pregnancy (Heilmann, 1986) and in oral contraceptive users (Heilmann *et al.*, 1981). Changes in the ability of erythrocytes to pass through the spleen as a result of changes in their membrane deformability would lead to an increase in splenic red pulp macrophage erythrophagocytic activity. To date no studies have been conducted that relate these changes in erythrocyte deformability to changes in macrophage erythrophagocytosis. The relevance of a physiological estrogen milieu to the normal function of these cells also has not been investigated. Does a marked decrease in endogenous estrogen levels lead to a decrease in both macrophage number and function?

Indomethacin's mechanism of blockade of the estradiol-induced splenic macrophage hyperplasia may represent a clinically important function for this NSAID. The primary action of the class of anti-inflammatory agents represented by indomethacin is blockage of the cyclooxygenase pathway of prostaglandin production. In the case of monocytic macrophages, inhibition of prostaglandin production by these cells results in

increased rather than decreased activity. Indomethacin's effect on the splenic red pulp macrophages may be mediated by a non-prostaglandin mechanism. Indomethacin has also been shown to have an effect on hematologic parameters. Mizushima and Sakai (1969) showed that *in vitro* incubation of washed erythrocytes with indomethacin resulted in changes in their fragility characteristics while Razin *et al.* (1981) reported that treatment of mice with indomethacin resulted in a significant decrease in hematocrit. As with estrogen, changes in erythrocyte membrane characteristics induced by indomethacin could lead to increased levels of splenic erythrophagocytosis and subsequent hematocrit decreases. The question also arises whether coadministration of the two drugs may produce an interactive effect resulting in an exacerbation or diminution of the erythrocyte changes occurring as a result of the presence of either drug alone.

Hypotheses

The present *in vivo* and *in vitro* studies were conducted to investigate the following hypotheses:

- Indomethacin's inhibition of estradiol-induced macrophage stimulation is mediated by a dose-related non-prostaglandin mechanism.
- Ovariectomy alone produces a decrease in the number of red pulp macrophages but does not have an effect on their phagocytic capabilities.
- The stimulatory action of estradiol on the red pulp macrophages is further enhanced by its direct damaging effect on erythrocyte membranes resulting in increased erythrophagocytosis. Indomethacin also contributes to increased erythrocyte removal by producing changes in membrane fragility.

Chapter 2

MATERIALS AND METHODS

In vivo NZW Rabbit Study

Drugs and Analytical Reagents.

Three-week constant-dose-release IN and E_2 pellets were obtained from Innovative Research (Toledo, OH). Tritium-labeled PGE_2 ([5,6,8,11,12,14,15-^{3}H(N)]-PGE_2) for determining PGE_2 recovery after tissue extraction was obtained from the New England Nuclear division of Dupont (Wilmington, DE). Indomethacin standard for determination of serum indomethacin levels was obtained from Sigma Chemical (St. Louis, MO, Cat # I 7378). Anesthetics used were; Ketaset® (ketamine hydrochloride) (Bristol Laboratories Veterinary Products, Syracuse, NY), Rompun® (xylazine) (Mobay Corp., Shawnee, KS) and pentobarbital sodium (Fort Dodge Laboratories, Fort Dodge, IA)

Animals.

Animals for this study were sexually mature (mean weight 3.45 kg ± 0.23), specific-pathogen-free (SPF), female New Zealand White (NZW) rabbits obtained from Myrtle's Rabbitry (Franklin, TN). The experimental protocol and housing and care of the rabbits were approved by the East Tennessee State University Committee on Animal Care (Project # 880401) following the National Institutes of Health and United States Department of Agriculture guidelines.

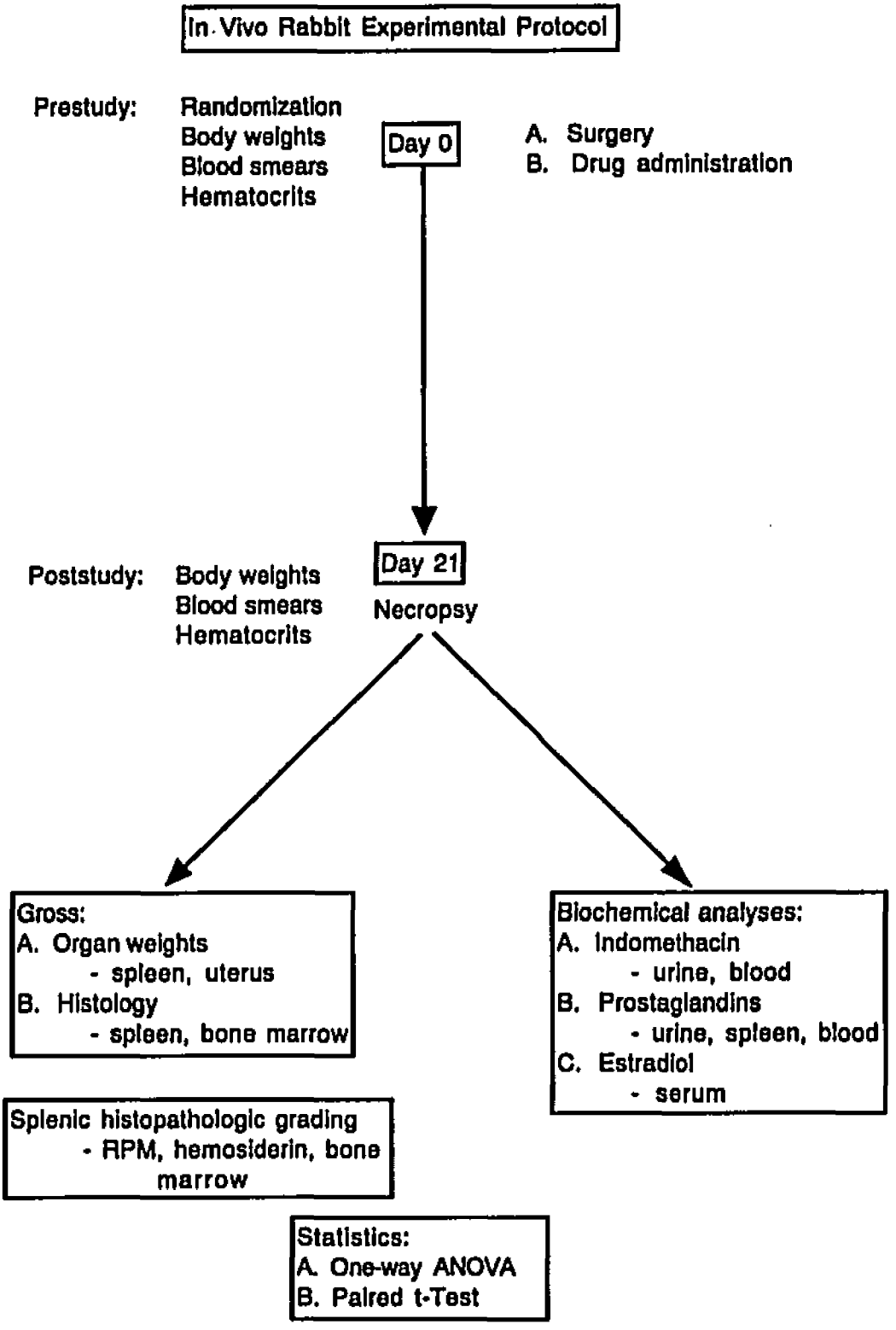
Experimental Design.

Thirty-nine rabbits were randomly assigned to 9 treatment groups of 4 animals each and a tenth control group (nontreated, intact Control) of 3 animals. The treatment groups were; 1.) Ovariectomized (OVX) Control, 2.) OVX plus IN at 0.1 mg/kg bw/day, 3.) OVX plus IN at 5.0 mg/kg bw/day, 4.) Sham OVX (SOVX) Control, 5.) SOVX plus IN at 0.1 mg/kg bw/day, 6.) SOVX plus IN at 5.0 mg/kg bw/day, 7.) OVX plus 25.0 mg E_2, 8.) OVX plus 25.0 mg E_2 plus 0.1 mg/kg bw/day IN and 9.) OVX plus 25.0 mg E_2 plus 5.0 mg/kg bw/day IN. The maximum IN dose used in this study was chosen to reduce the possibility of overt toxicity observed in some animals at the higher doses used in a previous study.

On the initial day of study body weights and hematocrits were obtained and all OVX treatment group animals were placed under general anesthesia (35.0 mg/kg body weight Ketaset® plus 7.0 mg/kg body weight Rompun® intramuscular, followed by ~ 1.0 ml pentobarbital sodium intravascular), ovariectomized and implanted subcutaneously with 3 week constant-dose-release pellets containing either IN or E_2, or both pellet types. The ovaries of the SOVX groups were manipulated and replaced at the time of drug implantation under general anesthesia. A schematic diagram of the experimental protocol is shown on page 16.

Necropsy.

At the end of the 21-day study period body weights and hematocrits were again obtained. All animals were then euthanized using an overdose of pentobarbital sodium and necropsied. At necropsy spleen weights were obtained, gross pathologic changes were noted and samples of blood, urine, spleen and bone were taken for further analyses.


In Vivo Rabbit Experimental Protocol
Prestudy:
Randomization
Body weights
Blood smears
Hematocrits
Day 0
A. Surgery
B. Drug administration
Poststudy:
Body weights
Blood smears
Hematocrits
Day 21
Necropsy
Gross:
A. Organ weights
- spleen, uterus
B. Histology
- spleen, bone marrow
Biochemical analyses:
A. Indomethacin
- urine, blood
B. Prostaglandins
- urine, spleen, blood
C. Estradiol
- serum
Splenic histopathologic grading
- RPM, hemosiderin, bone marrow
Statistics:
A. One-way ANOVA
B. Paired t-Test

Histopathology.

Sections of spleen were fixed in 10% neutral phosphate-buffered formalin (NBF), routinely processed and paraffin embedded. Sections were cut at 5 μm and stained with hematoxylin and eosin (H & E) stains and Prussian blue iron stain. Slides were evaluated blindly for splenic red pulp changes by two independent evaluators. Changes in red pulp macrophage populations were graded using a 0 to 4 grading scale, with 0 indicating minimal presence of macrophages and 4 indicating marked presence of macrophages. Cytoplasmic hemosiderin levels in red pulp macrophages (a measure of erythrophagocytosis) were evaluated in Prussian blue-stained spleen sections using the same grading scale, with 0 as an indicator of minimal cytoplasmic staining intensity and 4 indicating a high cytoplasmic staining intensity.

Sections of rib bone for marrow evaluation were taken from the costal-chondral region, fixed in NBF and decalcified in a mixture of 40% formic acid and Amberlite IR - 120 P ion exchange resin (Sigma Chemical Co., St. Louis, MO) for at least 24 hours. Following decalcification, the specimens were routinely processed for histology and stained with H & E stains. Bone marrow evaluations were based upon observed changes in overall cellularity, changes in megakaryocyte numbers and changes in numbers of myelocyte and erythrocyte precursors. Observed changes were graded from 0 to 4, with 0 indicating minimal cell numbers and 4 indicating marked cell numbers.

Hematocrits.

Microhematocrits were determined prior to the beginning of the study and at 21-days using the procedure of Strumia *et al.* (1954). Blood was drawn from an incision in the marginal ear vein of the rabbit into heparinized, 75 mm mirocapillary tubes. The tubes were spun for 2 minutes in a IEC MB Microhematocrit centrifuge (Damon/IEC

Division, Needham Heights, MA) and % hematocrit read using a Lancer® Spiracrit® microhematocrit reader (Lancer, St. Louis, MO). Each sample was run in duplicate and the results averaged.

Biochemical Analyses.

Estradiol Assays. Two ml aliquots of rabbit whole blood were collected in serum separation tubes (Becton-Dickinsen, Rutherford, NJ) at necropsy and the serum transferred to cryogenic storage vials (Nalge Co., Rochester, NY) which were kept frozen at -80°C until assayed.

Assay for E_2 serum levels was done using an acetylcholinesterase immunoassay system obtained from Cayman Chemical (Ann Arbor, MI)(ACE™EIA kits (Cat. # 582251)). All reagents were provided in the kit or prepared following instructions in the assay kit manual. Purification of serum samples prior to assay was accomplished using a liquid-liquid extraction procedure as follows; 1. 2 ml of deionized-distilled H_2O and 10 ml of diethyl ether were added to 20 ml test tubes containing 500 µl of serum, 2. test tubes were then vortexed thoroughly and allowed to stand for 5 minutes, after which time they were centrifuged for 10 minutes at 2000 x g., 3. the organic layer was transferred to a clean 10 ml test tube and the solvent evaporated under a stream of N_2, 4. the dried extract was then dissolved in 500 µl of EIA buffer. A standard curve was generated using duplicate 50 µl aliquots of eight different concentrations of the estradiol standard provided (range - 7.8 pg/ml to 1000 pg/ml). Each sample was run as 50 µl aliquots in duplicate at two different dilutions (1:5 and 1:10). Samples and standards were added to 96 well EIA plates prepared as per assay kit manual instructions. Additional wells were used to determine blank values (B), total activity (TA), non-

specific binding (NSB) and maximum binding (B_0). Estradiol acetylcholinesterase tracer (50 µl) was added to each well (except for the TA and B wells). The estradiol antiserum (50 µl) was then added to each well (except for TA, NSB and B wells).

The plates were then covered with aluminum foil, incubated for one hour on a Vortex Genie 2™ shaker (Fisher Scientific, Pittsburgh, PA) and developed using 200 µl of Ellman's Reagent (acetylcholinesterase substrate) per well (except for the TA wells which received 5 µl of tracer). Upon completion of development the plates were read at 412 nm on a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) and the results analyzed using SOFTmax® EIA analysis software (Molecular Devices, Menlo Park, CA). Sample E_2 concentrations were determined as follows; 1. average NSB was subtracted from average B_0 to determine corrected B_0, 2. the average NSB reading was then subtracted from the sample or standard well reading and divided by the corrected B_0, this figure was multiplied by 100 to yield $\%B/B_0$, 3. a linear regression curve for the estradiol standards was then generated using concentration as the X variable and $\%B/B_0$ as the Y variable, unknown sample concentrations were determined by entering $\%B/B_0$ as the Y value to determine the concentration X value.

Prostaglandin Assays. Samples of blood, urine and spleen for PGE_2 analyses were collected in silicon coated test tubes containing IN (25 µg/ml dissolved in ethanol) and added to phosphate buffered saline (PBS). The urine and blood samples were then frozen at -80°C while the spleen samples were homogenized in PBS with 10 second bursts of a Tekmar® Tissumizer® tissue homogenizer (Tekmar Company, Cincinnati, OH) and then frozen at -80°C. PGE_2 levels were determined using an ACE®-EIA assay kit (Cayman Chemical Co., Ann Arbor, MI, Cat# 514011). Urine samples were assayed

directly while blood samples and spleen homogenates were purified using a solid phase extraction procedure as follows; 1. a 500 µl sample aliquot was added to a clean test tube to which 5000 cpm of [^{3}H]-PGE_2 had been added, 2. 2 ml of ethanol were then added and the tube vortexed, 3. the samples were allowed to stand for 5 minutes at 4°C and then centrifuged for 10 minutes at 1500 x g, 4. the supernatant was then poured into a clean 10 ml tube, 8 ml of 0.1 M phosphate buffer (pH 4) added and the tube vortexed, 5. a Millipore® C-18 reverse phase Sep-Pak® cartridge (Waters Associates, Milford, MA) was then activated by rinsing with 5 ml of ethanol followed by 5 ml of distilled-deionized H_2O, 6. the sample was passed through the cartridge followed by 5 ml of distilled-deionized H_2O and 5 ml of HPLC grade hexane (J. T. Baker Inc., Phillipsburg, NJ), 7. the sample was then eluted with 5 ml of ethyl acetate containing 1% ethanol, 8. the eluted sample was dried under a stream of N_2 and resuspended in 1 ml of EIA buffer (from assay kit), 9. 500 µl of the sample was used for scintillation counting with the remaining 500 µl used for the EIA analysis.

Samples for scintillation counting were suspended in 2.5 ml of commercial scintillation cocktail (ScintiVerse™ I, Fisher Scientific, Pittsburgh, PA) and counted for 1 minute on a Beckman LS 9800 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA). Counting results were recorded in counts per minute (cpm) (counting efficiency = 33.0%). A recovery factor (RF) was calculated using the following formula:

$$RF = \frac{2 \times \text{cpm of sample}}{^3[H]\text{- } PGE_2 \text{ added to sample (cpm)}}$$

Enzyme immunoassay for sample PGE_2 content was conducted in the same way as

for the E_2 assay. All samples were run in duplicate at two concentrations with the following exceptions; 1. assayed sample concentrations were "undiluted" and "1:10 dilution", 2. PGE_2 standard was added at eight concentrations in a range of 3.9 pg/ml to 500 pg/ml and 3. plates were incubated for 18 hours prior to development. Calculations for $\%B/B_0$ and determination of PGE_2 in each sample were done as previously shown for E_2 determination. Calculation of final PGE_2 concentrations were done using the following formulas:

$$PGE_2 \text{ (pg) in extracted sample} = \frac{\text{Value from EIA (pg/ ml)}}{RF} - \text{added } {}^3[H] - PGE_2 \text{ (pg)}$$

$$\text{Total } PGE_2 \text{ in sample (pg/ ml)} = \frac{PGE_2 \text{ (pg) in extracted sample}}{\text{Volume of sample used for purification (ml)}}$$

Indomethacin Assays. Analysis of IN levels in serum samples was done using a spectrofluorometric procedure. Samples for analysis were extracted using a modification of the procedure of Kim *et al.* (1988). The procedure used is as follows; 1. 500 µl of serum (sample or spiked standard) was mixed with 10 ml of ACS grade methylene chloride in a 15 ml capped centrifuge tube and shaken vigorously for 20 minutes before being centrifuged at 2500 rpm for 5 minutes, 2. following centrifugation, the upper aqueous layer was aspirated and the organic layer was slowly evaporated to dryness at 60°C under a stream of N_2, 3. the residue remaining after drying was reconstituted in 3 ml of 0.01 N sodium hydroxide and the tube gently inverted for 10 minutes, 4. the tubes were then allowed to sit for 20 minutes prior to analysis. Analysis of IN levels was done using a Turner 430 spectrofluorometer (excitation 295 nm, emission 385 nm) (Turner Associates, Palo Alto, CA). A standard curve was generated using normal serum

spiked with IN. This curve was linear over a concentration range of 250.0 ng/ml to 1.0 µg/ml (r=.99).

Statistical Analyses.

Statistical analyses of the results of this study were done using the paired t-Test to compare pretreatment and 21-day results. Intergroup significance was evaluated using a one-way ANOVA with a Duncan New Multiple Range post-hoc test or a Kruskal-Wallis test. An α level of .05 was set for determining significance. Statistics were done using either StatView™ II (Abacus Concepts, Inc., Berkeley, CA) or SuperANOVA™ (Abacus Concepts, Inc., Berkeley, CA) computer software packages.

In vitro NZW Rabbit Erythrocyte Fragility Studies

Drugs and Analytical Reagents.

Water soluble 17ß-Estradiol (E_2) (17ß-estradiol encapsulated in 2-hydroxypropyl-ß cyclodextrin, Sigma Chemical Company, St. Louis, Mo.) was prepared in Dulbecco's Phosphate Buffered Saline (PBS) (pH = 7.4) (Gibco Laboratories, Grand Island, NY) and added to rabbit whole blood at a level of 500 pg/ml. Lyophilized indomethacin sodium trihydrate (IN) (INDOCIN® I.V., Merck Sharp & Dohme, West Point, PA) was dissolved in PBS to a concentration of 1 mg/ml and added to rabbit whole blood at a level of 9.6 µg/ml. Stock buffered sodium chloride for the osmotic and mechanical fragility assays was prepared by dissolving 90.0 gm NaCl, 13.65 gm Na_2HPO_4 and 2.43 gm $NaH_2PO_4 \cdot H_2O$ in 1 liter of distilled water and the pH adjusted to 7.4 with 2N NaOH (chemicals from Fisher Scientific, Pittsburgh, PA). Citrate-Phosphate-Dextrose (CPD) anticoagulant/preservative (327.0 mg citric acid, 2.55 gm dextrose and 222.0 mg $NaH_2PO_4 \cdot H_2O$ in 100 ml of distilled/deionized water -

chemicals from Fisher Scientific, Pittsburgh, PA) was added to each blood collection tube.

Animals.

The animals used for bleeding were sexually mature, female, SPF NZW rabbits obtained from Myrtle's Rabbitry (Franklin, TN). The experimental protocol and housing and care of the rabbits were approved by the East Tennessee State University Committee on Animal Care (Project # 940104) following the National Institutes of Health and United States Department of Agriculture guidelines.

Drug Concentration Selection.

The E_2 dose for the osmotic and mechanical fragility assays was selected based upon the mean value obtained from the sera of the E_2-supplemented OVX groups in the *in vivo* study which was approximately 500 pg/ml. The IN dose was selected based upon constant infusion of a 5 mg/kg bw/day dose over a three-week period and was calculated as follows:

1. Given that the mean weight of animals receiving the high dose of IN was 3.49 kg, then each animal received approximately 17.44 mg/day (5 X 3.49) or 726.7 µg/hr which equals the infusion rate (IR).

2. Average concentration $(C_p) = \frac{1.44\ (t_{1/2})(f)(IR)}{V_d}$

Where $t_{1/2}$ for IN = 2.4 hr., f (fractional absorption) = 1, IR = 726.7 µg/hr, V_d (Volume of distribution) = 0.26 l/kg

Final value of C_p = 9.6 µg/ml of blood

Blood Collection.

Blood was collected from the marginal ear veins of 2 female, specific-pathogen-free, New Zealand White rabbits (which had received 0.1-0.2 cc of the analgesic/tranquilizer INNOVAR-VET® (Pitman-Moore, Inc., Washington Crossing, NJ) intramuscularly) using a vacuum bleeder (Bellco Glass, Inc., Vineland, NJ). Each animal was bled on alternate days. Aliquots of 10 ml were bled into sterile borosilicate tubes containing 1.4 ml of CPD. Blood was maintained at 39.5°C and used within one hour after collection.

Osmotic Fragility Assay.

Assay Procedure. Erythrocyte osmotic fragility was determined using a modification of the method of Dacie (1954). A 50 µl aliquot of rabbit whole blood at 39.5°C (mean rabbit body temperature) was added to each of twelve, 16 X 100 mm, borosilicate culture tubes containing 5 ml of either 0.85, 0.75, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20 or 0.10 percent NaCl and to one tube containing 5 ml of distilled/deionized water. The tubes were briefly vortexed (Vortex Genie 2™, Fisher Scientific, Pittsburgh, PA) and incubated for 30 minutes at 39.5°C (all incubations were done in a Stabil-Therm® Dry Type Bacteriological Incubator (Blue M Electric Co., Blue Island, IL)). Following incubation, the tubes were centrifuged for 5 minutes at 2000 rpm in an IEC DPR-6000 centrifuge (Intl. Equip. Corp., Needham Hts., MA). The amount of solubilized hemoglobin resulting from erythrocyte rupture was measured by absorbance readings in a Shimadzu UV160U UV-VIS dual beam spectrophotometer (Shimadzu Corp., Kyoto Japan) at a wavelength of 545 nm, using the supernatant from the 0.85% NaCl tube as the blank. The absorbances obtained were then plotted on a curve versus the NaCl concentrations.

Results Analysis. Absorbance results for the osmotic fragility assays were analyzed using a mathematical model based upon an assumed Gaussian distribution for the plasma membrane strengths for the erythrocyte population in each assay. The derivation of this model is discussed at length by Orcutt *et al.* (1995) and will not be addressed here. The absorbance (Abs) model used is represented by the following equation:

$$Abs = p3 \; erfc \left(\frac{x - p1}{p2} \right)$$

where *x represents the applied osmotic stress (measured as NaCl concentration)*, p1 is the location of the maximum point for the distribution function (measured as NaCl concentration), p2 is the width of that distribution function (measured as NaCl concentration), p3 is an experimentally determined measure of the number of erythrocytes present (measured as maximum erythrocyte hemolysis absorbance) and is constant for a given sample and erfc is the complimentary error function.

Mechanical Fragility Assay.

Erythrocyte mechanical fragility evaluation was conducted using a modification of the procedure of McPherson et. al. (1986). Twenty-five µl of rabbit whole blood (in triplicate) at 39.5°C was added to screw-capped, 13 X 100 mm, borosilicate tubes containing 5 ml of 1% NaCl (1:10 dilution of stock sodium chloride solution). Each tube (except for spontaneous hemolysis tubes) contained three-3 mm diameter, soda-lime glass beads (Fisher Scientific, Pittsburgh, PA). Following whole blood addition, the tubes were allowed to stand for 15 minutes at 39.5°C and then placed on a tube rocker (Thermolyne Corp., Dubuque, IA) for 90 minutes at 39.5°C. The tubes were then centrifuged for 10 minutes at 2000 rpm and the supernatants read at a wavelength of 545 nm to determine the supernatant hemoglobin optical density (SOD). The red blood

cell pellet remaining in the tubes was hemolyzed by adding 20 µl of a 10% solution of Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) and vortexing the tubes for 10 seconds. The hemolyzed pellet was then added to the supernatant and this solution was read at 545 nm to determine the total optical density (TOD). Percent hemolysis was determined by dividing the SOD by the TOD and multiplying by 100 (SOD/TOD X 100). Hemoglobin release due specifically to mechanical stress was determined by subtracting the mean percent hemolysis of the spontaneous hemolysis tubes (no beads) from the percent hemolysis values for the tubes containing glass beads.

Experimental Protocol.

Experimental protocols were initiated within an hour after blood collection. A 2 ml aliquot of rabbit whole blood was added to each of four sterile borosilicate tubes containing PBS alone, PBS plus 500 pg/ml E_2, PBS plus 9.66 µg/ml IN (2.7×10^{-5} M) or 500 pg/ml E_2 plus 9.66 µg/ml IN. Osmotic and mechanical fragility assays were done for each tube at the time of blood addition (i.e., initial results). Microcapillary tube packed cell volumes (PCV) were also obtained shortly after the blood was added to each tube (using the method of Strumia *et al.* (1954), see page 18). All tubes were then incubated at 39.5°C for four hours prior to conducting a second set of osmotic and mechanical fragility assays (i.e., final results) and obtaining post-incubation PCV's. Each experiment was repeated eight times using freshly obtained blood. A schematic diagram of the experimental protocols is shown in the flow chart on page 28.

Statistical Analyses.

An α level of .05 was set for determining significance for all statistical tests. Statistics were obtained using either StatView™ II (Abacus Concepts, Inc., Berkeley,

CA) or SuperANOVA™ (Abacus Concepts, Inc., Berkeley, CA) computer software packages.

Osmotic Fragility Results. Mathematically computed results for p1 and p2 from each experiment were averaged for each treatment and compared using a two-way ANOVA based upon the independent variables treatment and time. Pair-wise comparisons using a t-Test of least squares mean values were done for each variable when the ANOVA indicated significant treatment variation.

Mechanical Fragility Results. Hemolysis results of the initial and final mechanical fragility experiments were averaged and the mean values were tested using a one-way ANOVA based upon treatment. A Tukey-Kramer HSD post-hoc test was done when the ANOVA indicated significant treatment variation.

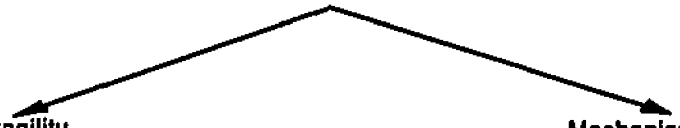
Osmotic and Mechanical Fragility Experimental Protocols

Rabbit blood collected from marginal ear vein into CPD anticoagulant/preservative

Experimental design:

1. Control (2 ml whole blood + 288.6 µl PBS)
2. Indomethacin (2 ml whole blood + 38.6 µl of 9.6 µg/ml IN + 250 µl PBS)
3. Estradiol (2 ml whole blood + 250 µl of 500 pg/ml E2 + 38.6 µl PBS)
4. Indomethacin + Estradiol (2ml whole blood + 38.6 µl of 9.6 µg/ml IN + 250 µl of 500 pg/ml E2)

Assays were run at time of drug addition and 4 hours later, after incubation at 39.5°C.



Osmotic Fragility

Procedure:

1. 12 tubes/assay containing 0.85 to 0.1% NaCl + a DI water tube
2. 50 µl treated blood added per tube, incubated at 39.5°C for 30 minutes
3. Centrifuge for 10 min. @ 2000 rpm
4. Supernatant read @ 545 nm to generate an optical density curve for each regimen

Mechanical Fragility

Procedure:

1. 25 µl blood from each regimen added to each of 3 tubes containing 3-3 mm glass beads, a set of control tubes contain no beads
2. tubes rocked for 1.5 hrs. @ 39.5°C, centrifuged and supernatant read @ 545 nm to generate supernatant optical density (SOD)
3. total optical density (TOD) determined by adding Nonidet P-40 to pellet, combining with supernatant and reading at 545 nm.
4. percent hemolysis = SOD/TOD X 100
5. mean control value subtracted from treatment value to determine hemolysis due to mechanical fragility

Chapter 3.

RESULTS

In vivo NZW Rabbit Study

Spleen Weights.

Mean weights of spleens for the OVX/E_2/IN 0.1 and OVX/E_2/IN 5.0 groups were significantly greater than the spleen weights for their respective control groups in the OVX and SOVX treatment regimens and for the intact Control group (Figure 1). Mean spleen weight values for animals in the OVX Control and OVX/IN groups were not significantly different from their respective SOVX control groups, nor were they significantly different from the mean value for the intact Control group.

Histopathologic Evaluations.

Correlations in grading of histologic changes for the two evaluators were r = .925 for RPM grade and r = .985 for hemosiderin staining grade.

Splenic red pulp macrophages. The histologic appearance of the red pulp in RPM grade 0 spleens consisted of numerous well-defined, slightly dilated venous sinuses and thin splenic cords with few macrophages. With higher grade the architecture became increasingly disrupted as the cords became thicker and macrophages more numerous, often forming whorled nests of cells. The venous sinuses in these higher grades became less abundant and more dilated. Grade 4 spleens often contained areas of red pulp which consisted of sheets of macrophages with little or no evidence of normal venous sinus organization. Representative spleen sections for the 0 to 4 grading scale used to evaluate changes in splenic RPM grades are shown in Plates 1a - 1e.

Ovariectomy produced an overall significant decrease ($p<.05$) in numbers of splenic cord RPM when comparison was made to the SOVX and E_2-supplemented treatment regimens and to the Intact Control group. Supplementation with E_2 increased the RPM grade over that of the OVX regimen although not to a level which was significantly greater than that of the Intact Control group. The addition of IN was effective in decreasing the RPM grade in the SOVX/IN 5.0 group when compared to its respective control in the OVX/E_2 treatment regimen (OVX/E_2/IN 5.0). (Figure 2)

RPM hemosiderin staining intensity. Evaluation of hemosiderin levels in the splenic RPM indicated a low level of staining in the spleens of the OVX E_2-supplemented groups with the mean grades of the OVX/E_2 Control and OVX/E_2/IN 0.1 levels being significantly different from that of the Intact Control group (Figure 3). The remaining groups hemosiderin staining levels were not significantly different from their corresponding control groups or from that of the Intact Control group. An example of a hemosiderin grade 4 spleen section is shown in Plate 2.

Bone marrow evaluations. No significant differences were found among any of the groups in evaluations of overall bone marrow cellularity, numbers of erythroid precursors, myeloid precursors or megakaryocytes ($p<.05$).

Hematocrits

The pretreatment mean hematocrits for each group ranged between 39% and 45%. The 21-day mean values ranged between 36% for the OVX/E_2/IN 5.0 group and 41% for the SOVX/C group. Indomethacin treatment at 5.0 mg/kg bw/day resulted in a significantly reduced 21-day mean hematocrit ($p<.05$) in the OVX group (Figure 4).

Estradiol supplementation also resulted in reduced mean 21-day hematocrits and addition of IN at 5.0 mg/kg bw/day exacerbated the E_2 effect.

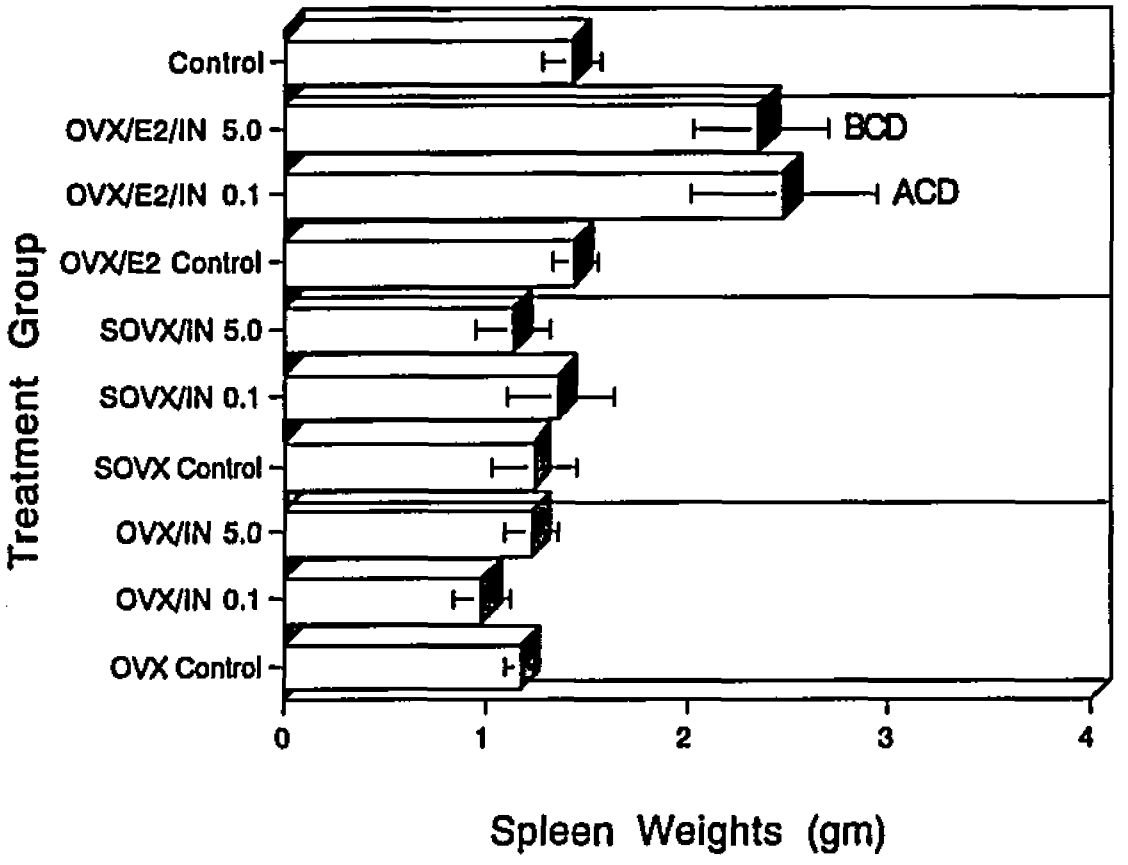


Figure 1. Mean spleen weight intergroup comparisons (±SEM). A-Significantly different from OVX/IN 0.1 and SOVX/IN 0.1 groups ($p<.05$), B-Significantly different from OVX/IN 5.0 and SOVX/IN 5.0 groups ($p<.05$), C-Significantly different from OVX/E_2 Control group ($p<.05$), D-Significantly different from Intact Control group ($p<.05$) ($n = 4$, except for Intact Control where $n = 3$).

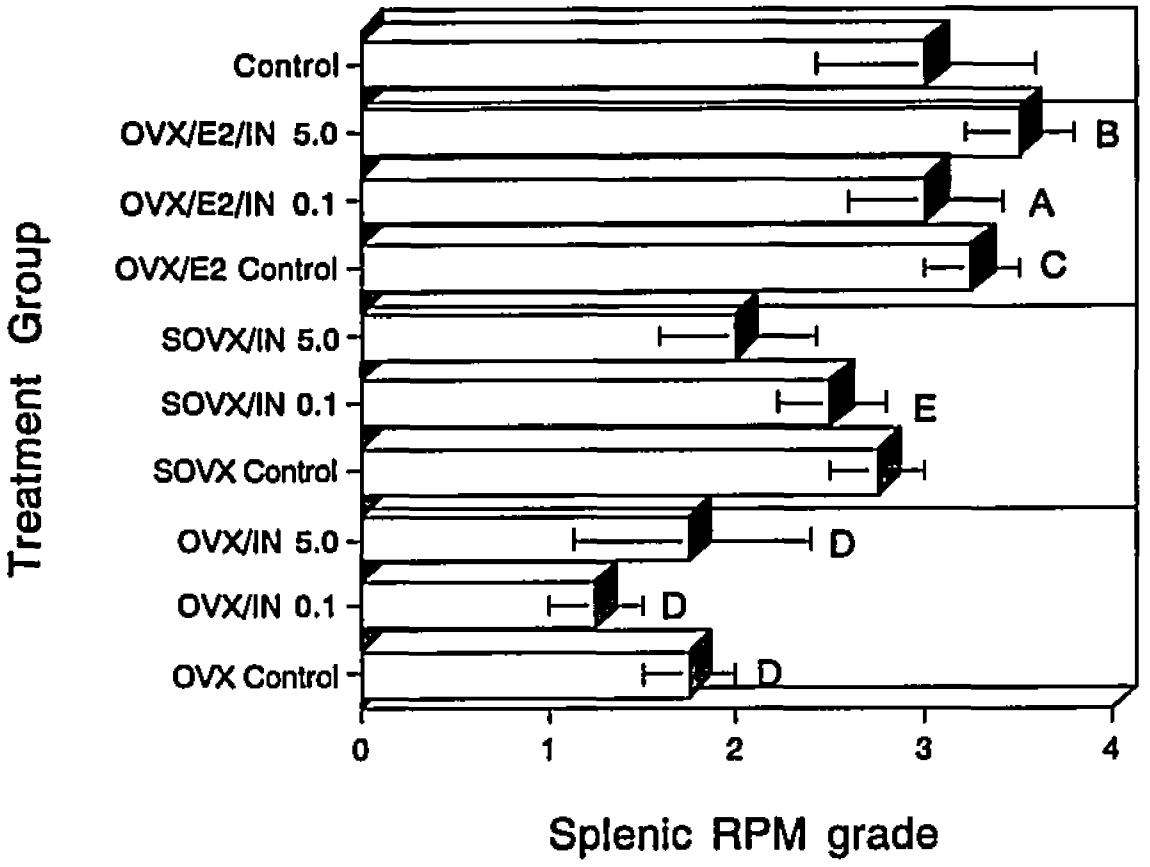
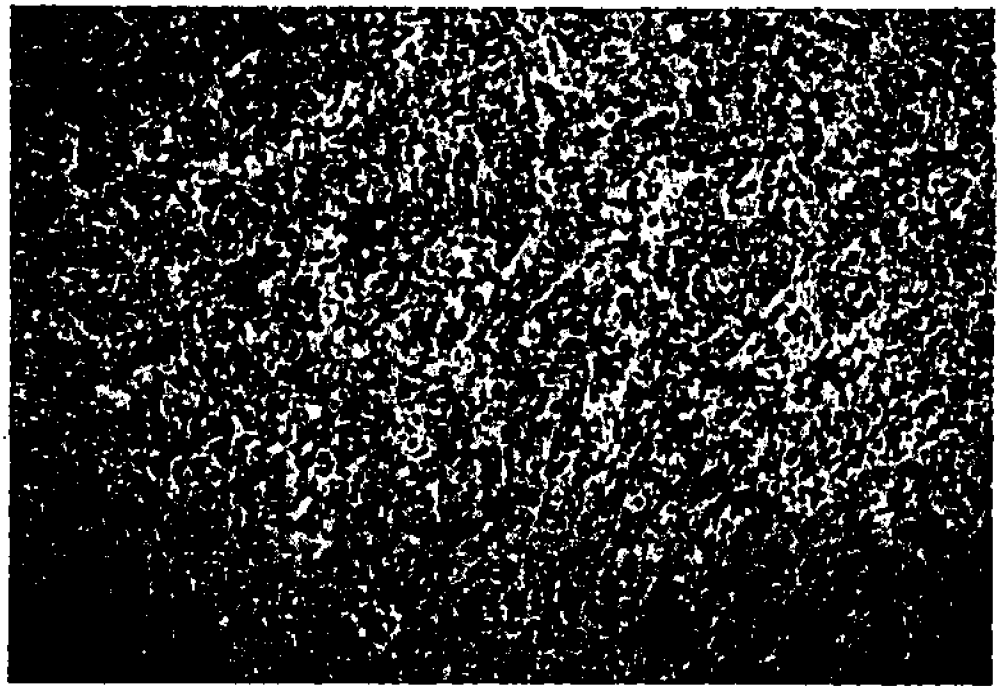


Figure 2. Intergroup significance of mean splenic RPM grade (±SEM). A-Significantly different from OVX/IN 0.1 group ($p<.05$), B-Significantly different from OVX/IN 5.0 and SOVX/IN 5.0 groups ($p<.05$), C-Significantly different from OVX Control group ($p<.05$), D-Significantly different from intact Control group ($p<.05$), E-Significantly different from OVX/IN 5.0 group ($p<.05$) ($n = 4$, except for intact Control group where $n = 3$).



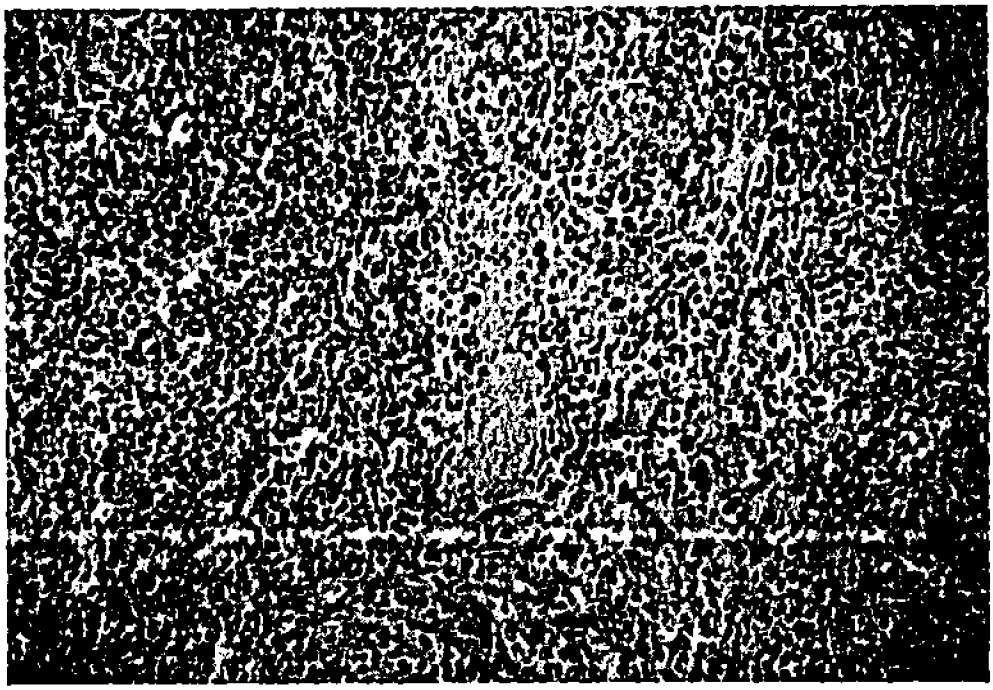
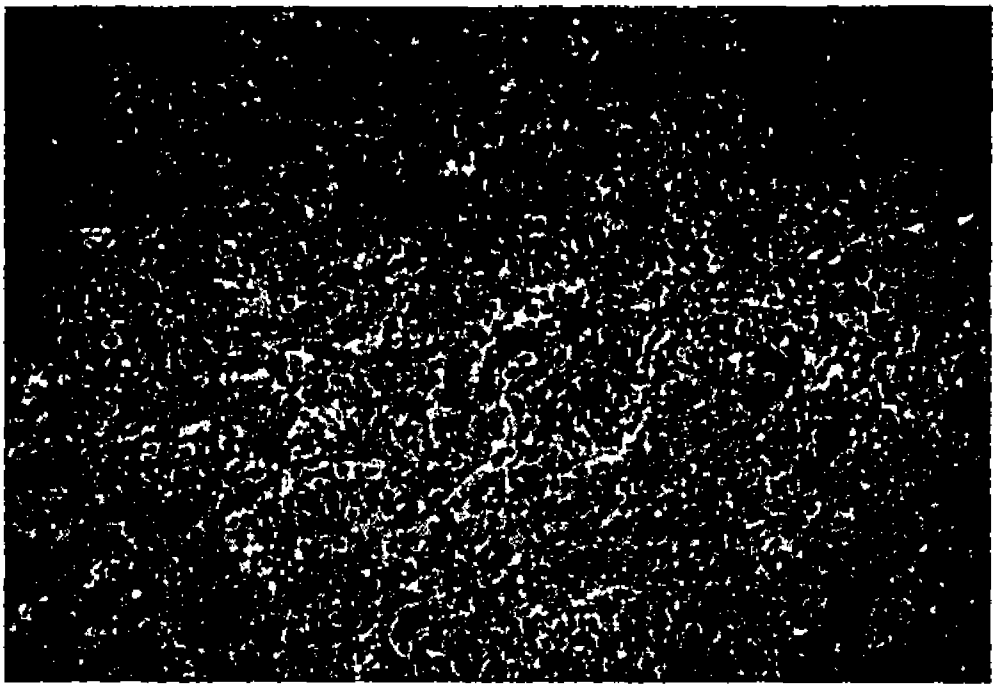


Plate 1a (Top). Photomicrograph of grade 0 rabbit splenic red pulp with well-defined venous sinuses (short arrow) and thin splenic cords (long arrow). H & E., X 160.

Plate 1b (Bottom). Grade 1 splenic red pulp. H & E., X 160



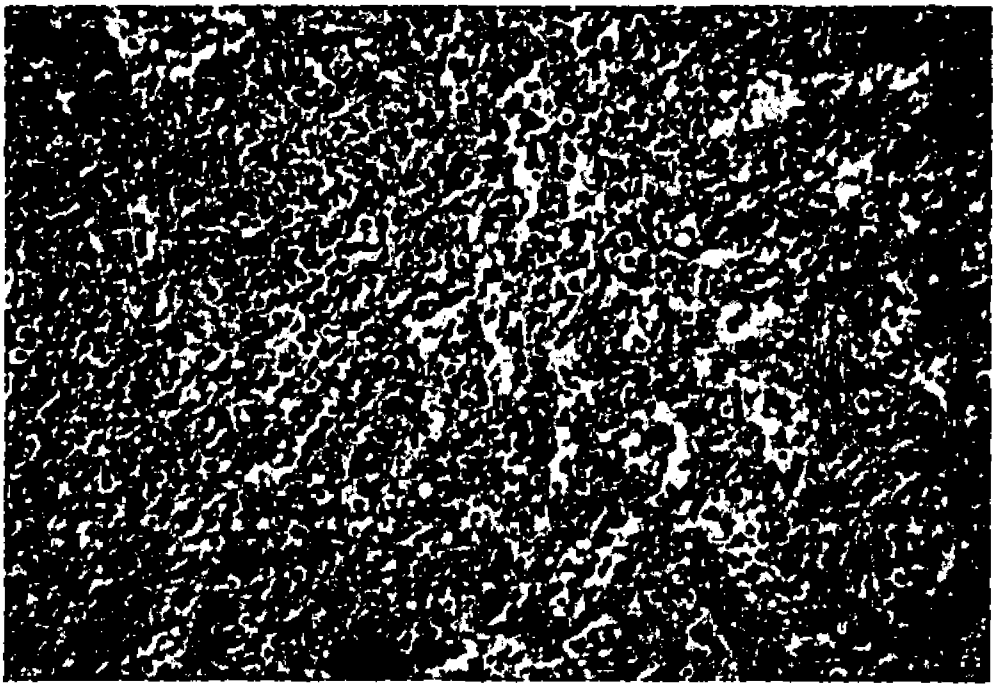


Plate 1c (Top). Grade 2 splenic red pulp. H & E., X 160

Plate 1d (Bottom). Grade 3 splenic red pulp. H & E., X 160

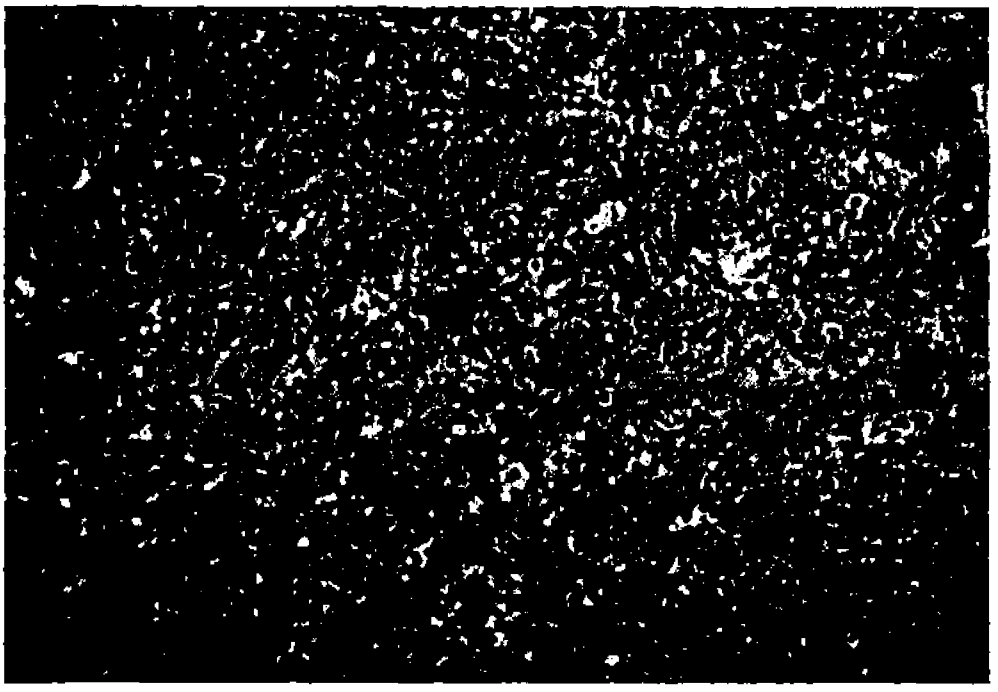


Plate 1e. Grade 4 splenic red pulp, note greater loss of architecture with greatly thickened splenic cords and few venous sinuses. H & E., X 160.

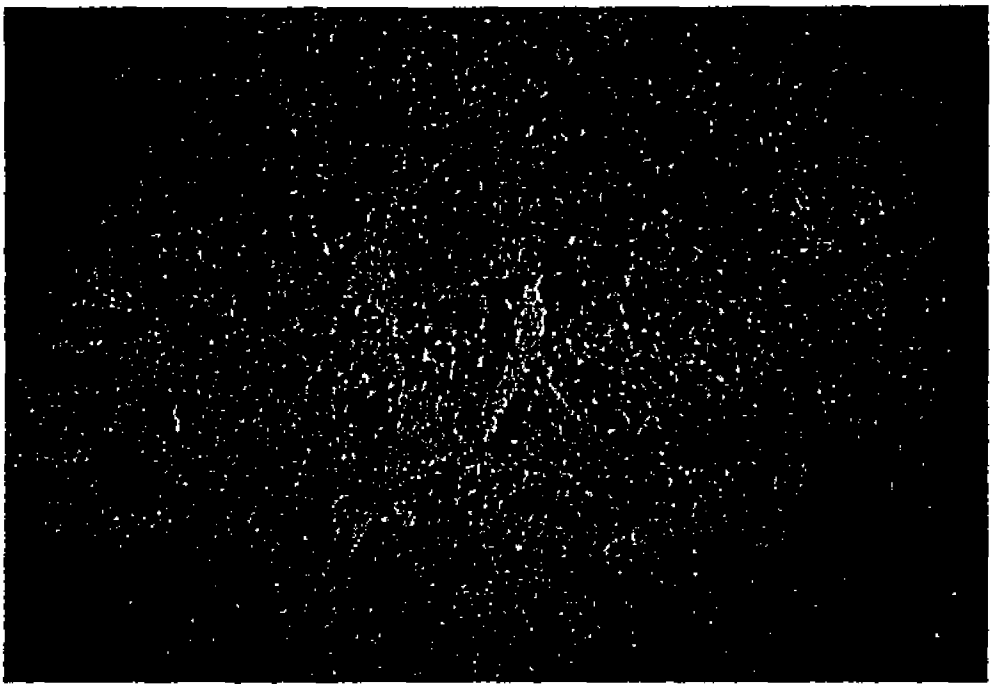


Plate 2. Photomicrograph of hemosiderin laden splenic RPM in a grade 4 spleen (OVX Control animal). Prussian blue iron stain, X 200

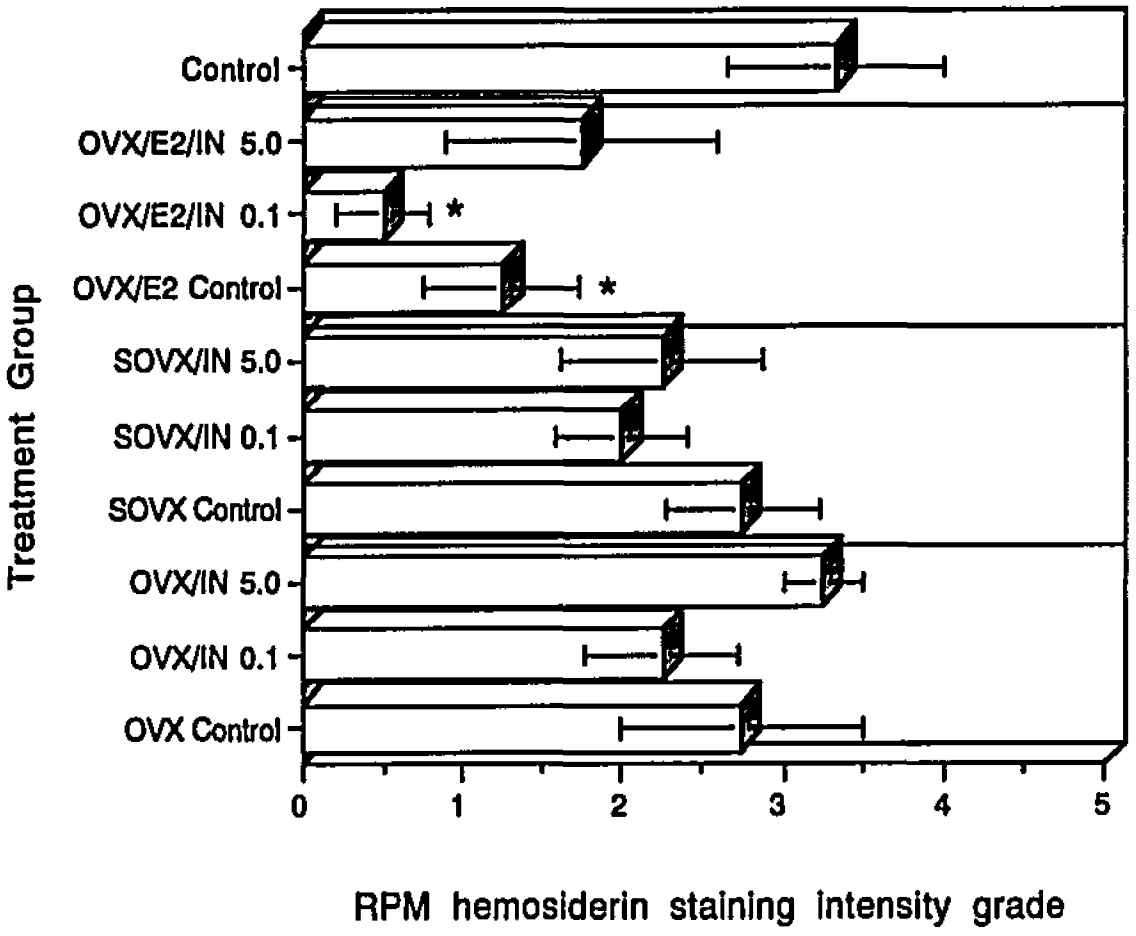


Figure 3. Intergroup comparison of mean splenic RPM hemosiderin staining intensity grades (±SEM). (*Significantly different from Intact Control group level, $p<.05$) ($n = 4$, except for Intact Control group where $n = 3$).

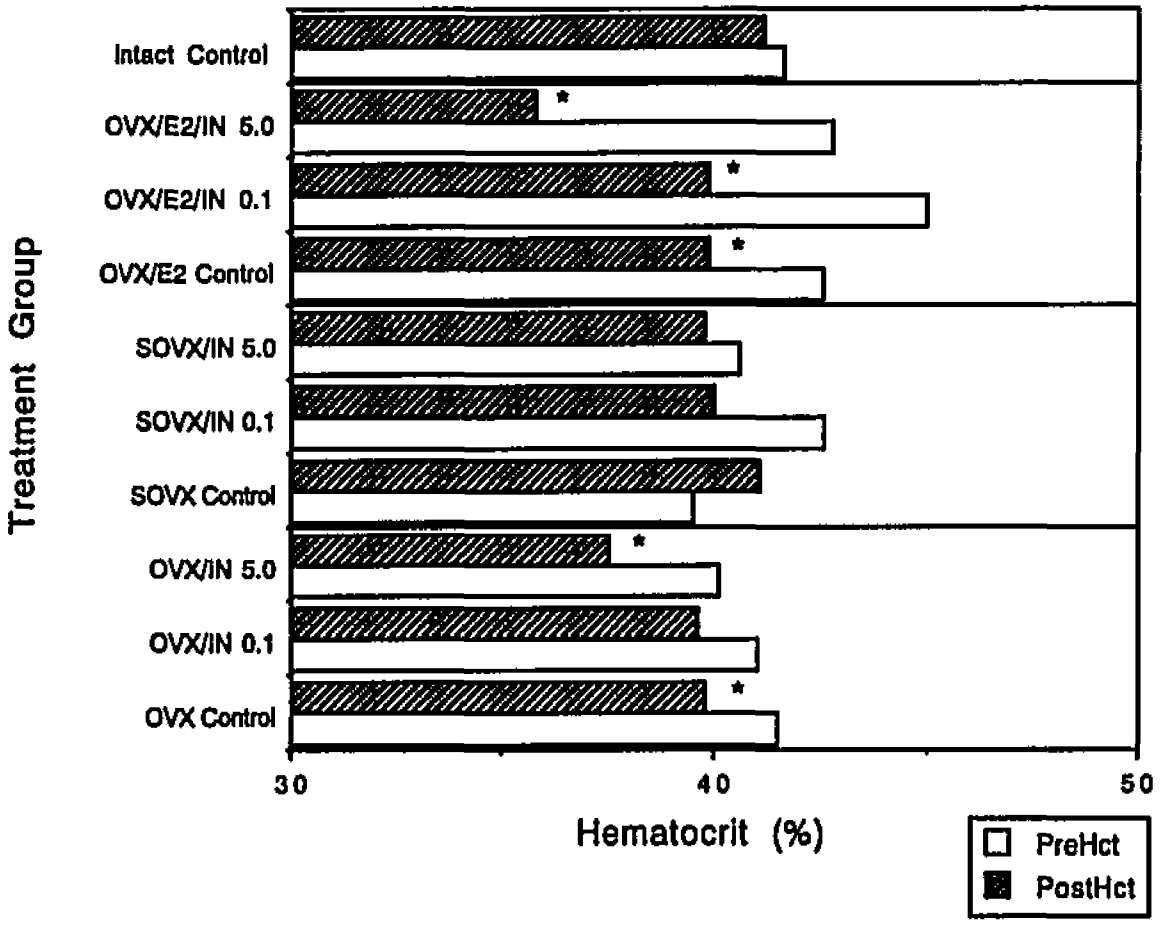


Figure 4. Comparison of pretreatment and 21-day mean hematocrits (*Significantly different from pretreatment value, $p<.05$) ($n = 4$, except for Intact Control group where $n = 3$).

Biochemical Analyses.

Serum estradiol levels. Mean serum levels of estradiol were significantly elevated in the OVX/E_2-supplemented groups (Figure 5) when compared to the OVX non-supplemented group. The levels of E_2 in the SOVX groups and the Intact Control group were highly variable and are not shown. The cause for this high variability may be related to the fact that although rabbits are induced-ovulators they do display an estrus cycle with marked changes in estrogen levels on a day-to-day basis (Batra and Källstrand, 1979).

Prostaglandin E_2 levels. Prostaglandin E_2 levels were not significantly different for any of the tissues analyzed .

Indomethacin levels. Levels of IN in blood and urine from the 0.1 and 5.0 mg/kg bw/day groups were below the detectable range of the assay.

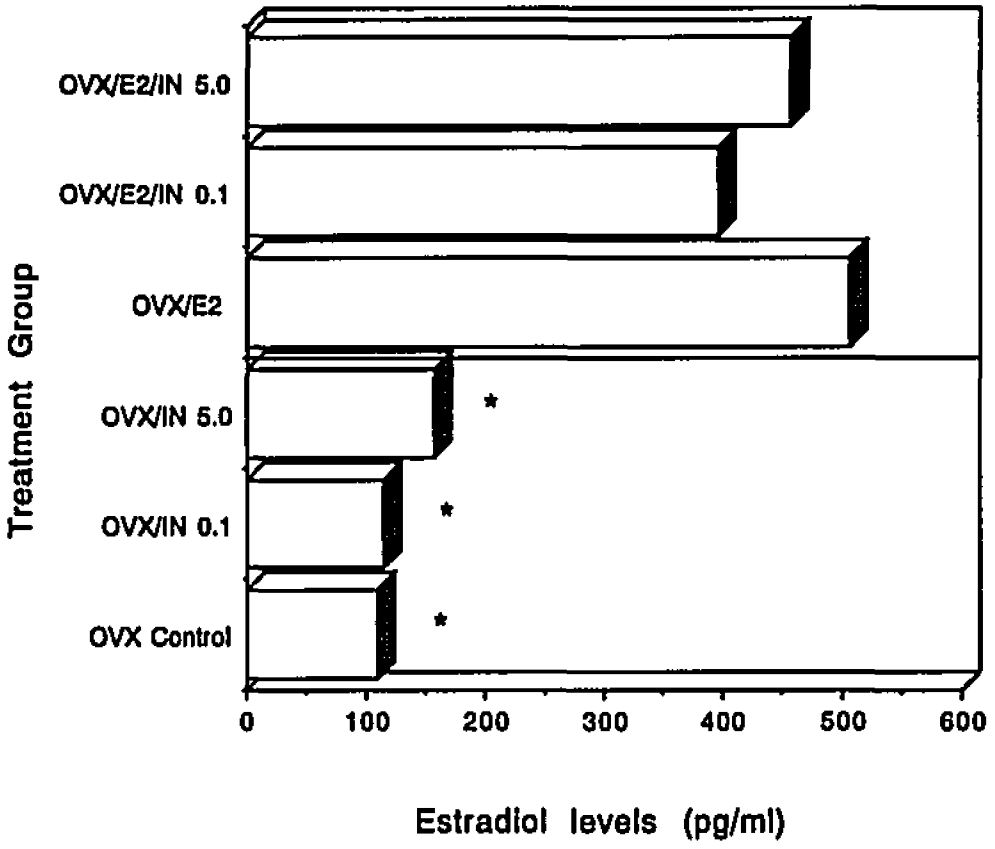


Figure 5. Intergroup significance for serum levels of E_2 in OVX and OVX/E_2-supplemented groups (standard error bars not shown due to high level of intra-group variability). (*Significantly different from respective OVX/E_2-supplemented control group.)

In vitro NZW Rabbit Erythrocyte Studies

Osmotic Fragility Experiments.

The results for the osmotic fragility experiments are shown in Figure 6 (normalized initial osmotic fragility curve, Figure 6; normalized final osmotic fragility curve, Figure 7). Statistical analyses of the mean values for p1 (50% hemolysis point) indicated that there was no significant difference between the p1 values for the 4 treatment groups at the initial measurement. Analyses of the final results showed that the mean p1 values for the Control and E_2 treatments were significantly different from those for the IN and IN plus E_2 treatments. This significance indicates a greater degree of hemolysis for the IN and IN plus E_2 treated erythrocytes when compared to Control and E_2 treated erythrocytes at the same NaCl concentration. Comparison between initial and final mean p1 values for each treatment indicated that a significant incease in osmotic fragility for IN and IN plus E_2 treated erythrocytes occurred between the initial measurement and the final measurement (Figure 8).

Analyses of the p2 (hemolysis dispersion) mean values indicated that no significant difference among the treatments existed at the initial measurement or after 4 hours. A significant change did occur for the p2 values for IN and IN plus E_2 treated erythrocytes between the initial and final measurements (Figure 9). This finding indicates that an increase in population susceptibility to osmotic stress occurred for the erythrocytes in these two treatments.

Mechanical Fragility Experiments.

The results of the 8 mechanical fragility experiments are shown in Figure 10. There was no significant difference in mean MF values between the treatments at the

initial measurement. The mean levels of hemolysis for the IN, E_2 and IN plus E_2-treated samples at the final measurement were all greater than that of the Control at that time. The effect of E_2 in the IN plus E_2 regimen was neither additive nor synergistic with the IN induced hemolysis. It must also be noted that E_2 did not have a diminutional effect in the results from the IN plus E_2 regimen.

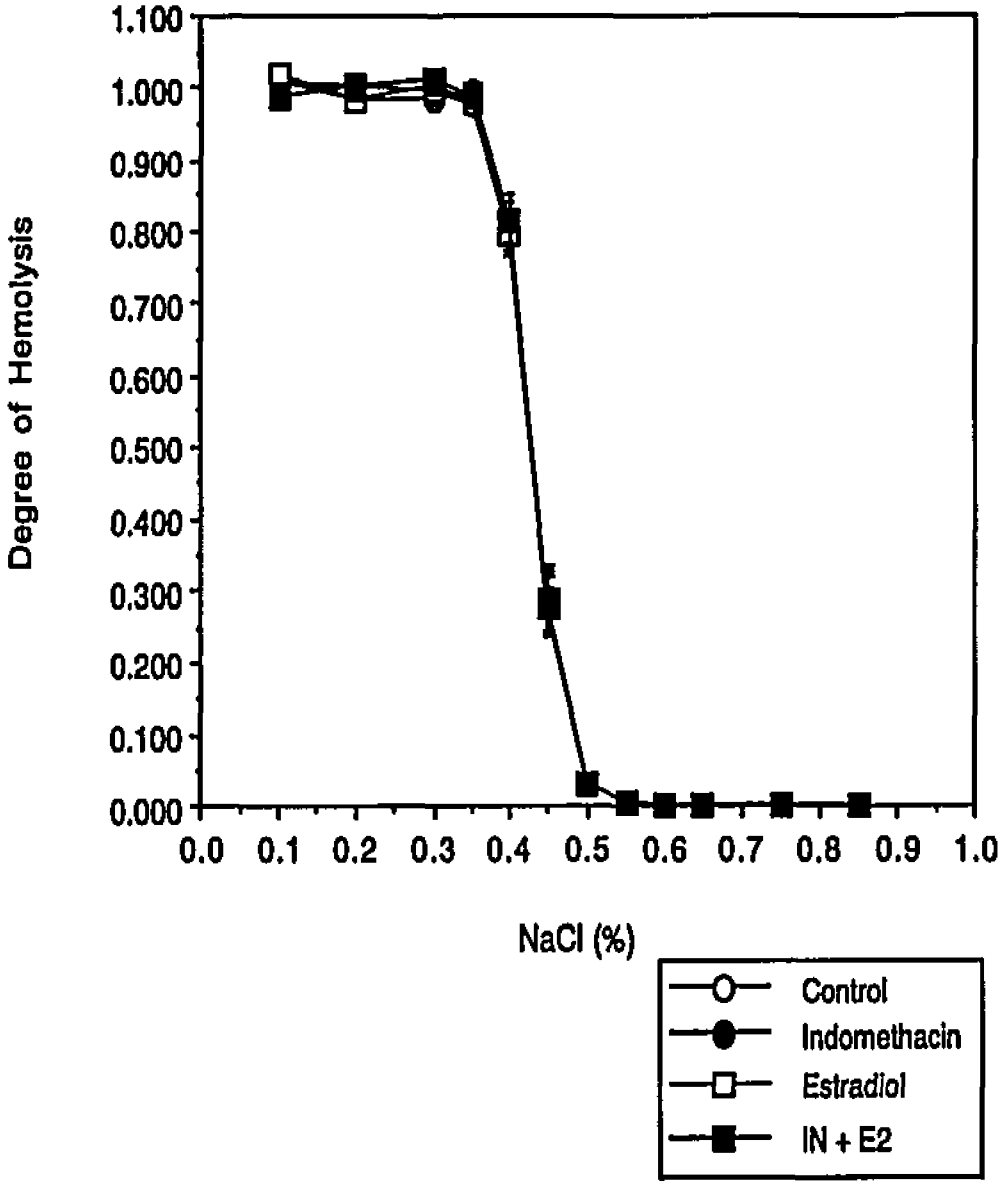


Figure 6. Normalized values for osmotic fragility results at time of drug addition (initial results) (mean of 8 experiments ± SEM)

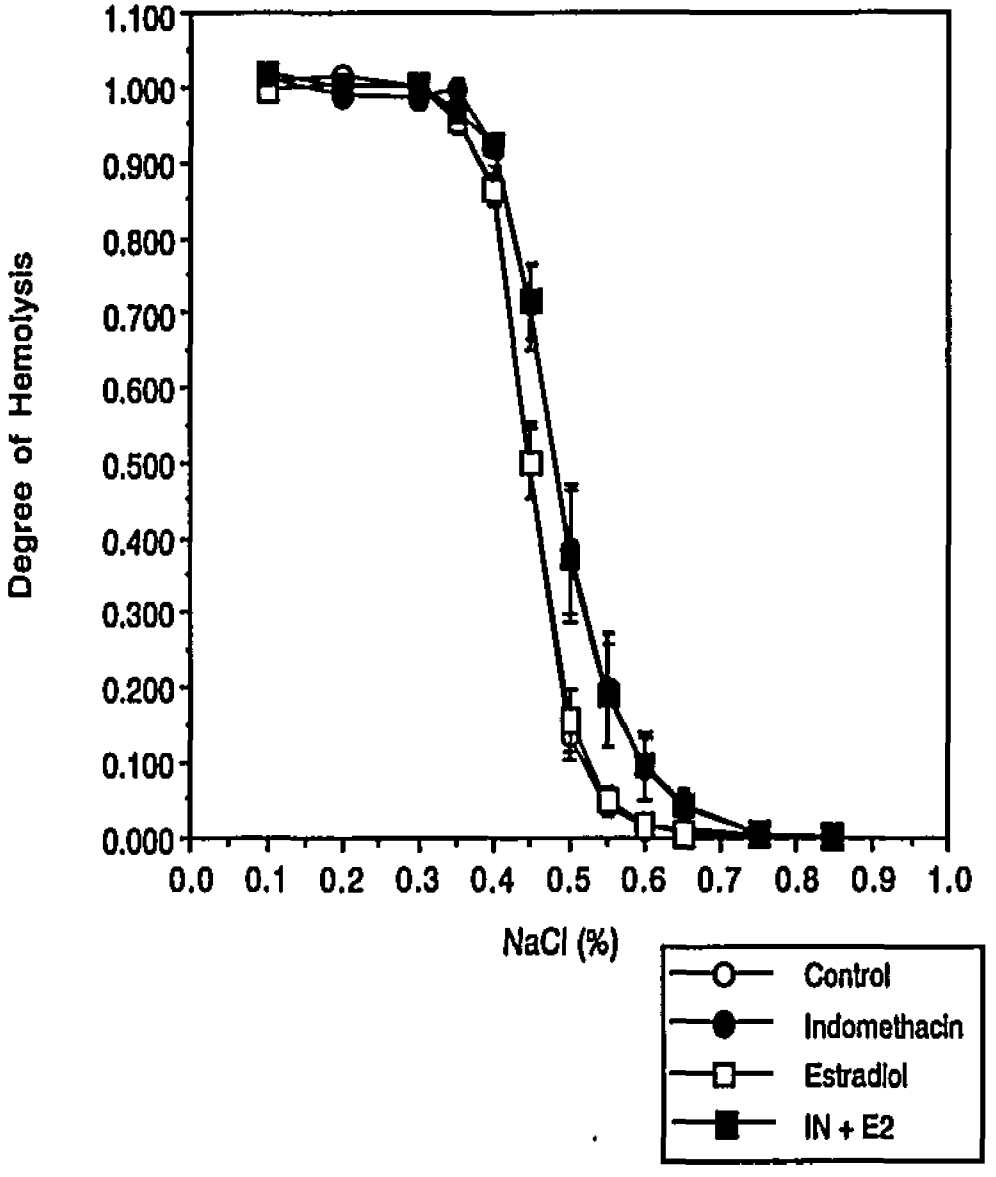


Figure 7. Normalized values for osmotic fragility results at 4 hours (final results) (mean of 8 experiments ± SEM)

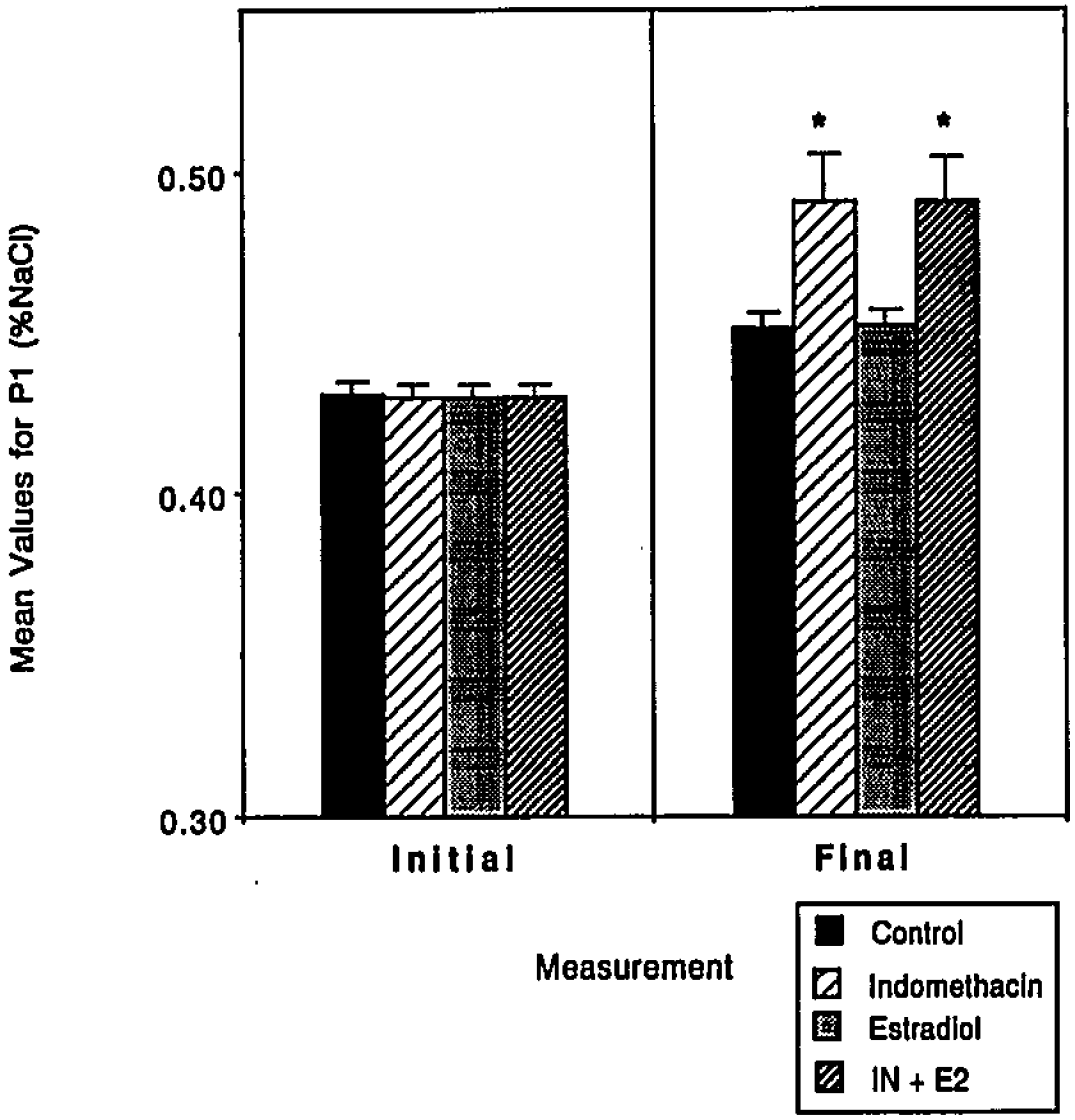


Figure 8. Comparison of mean NaCl concentrations (± SEM) at which 50% hemolysis (p1) occurred over time for each treatment regimen (*Significantly different from initial treatment regimen and final Control value, $p<.05$) ($n = 8$).

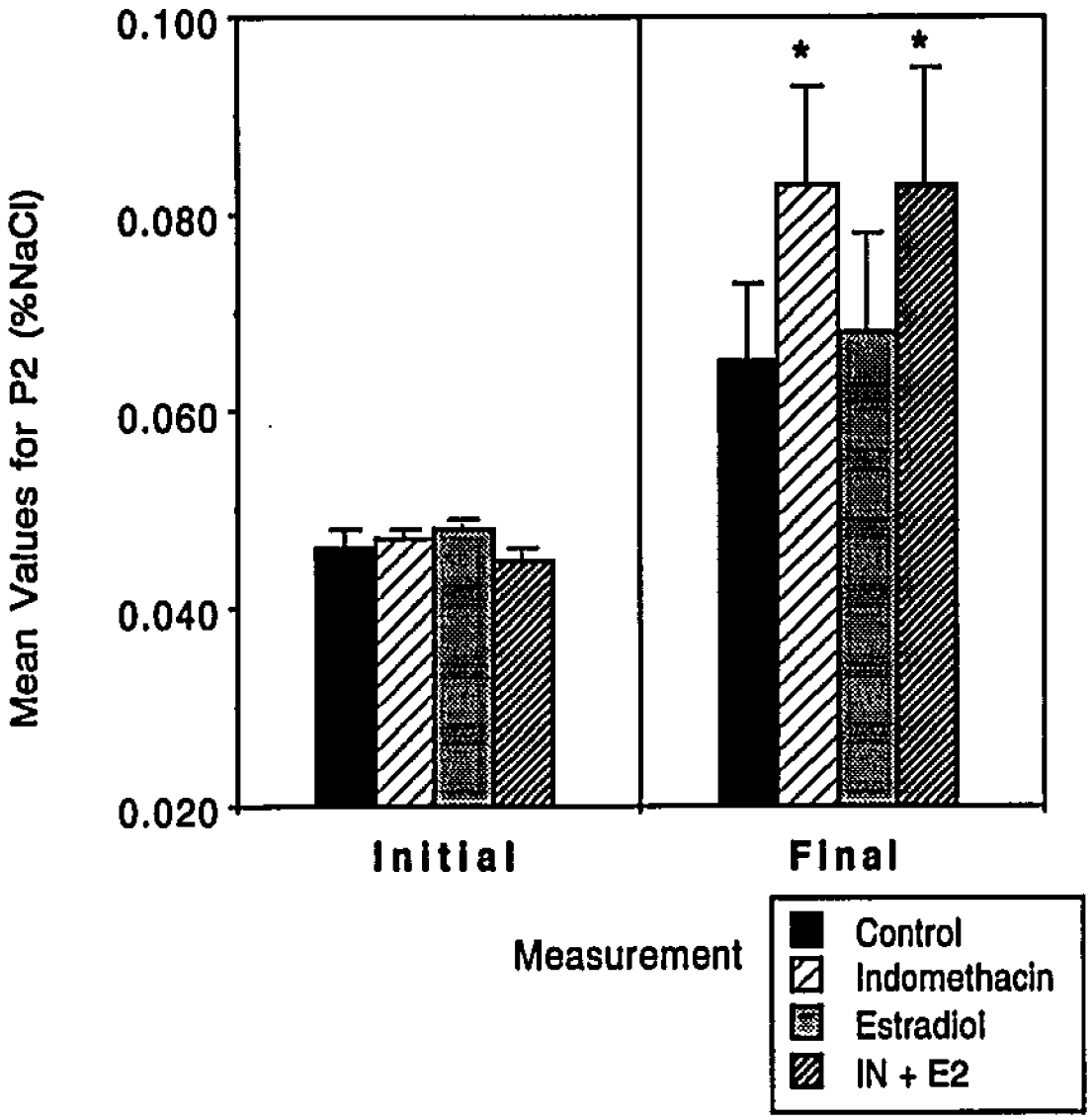


Figure 9. Comparison of mean NaCl concentrations (± SEM) for dispersion of erythrocyte hemolysis (p2) for each treatment regimen over time (*Significantly different from initial treatment regimen values. $p<.05$) ($n = 8$).

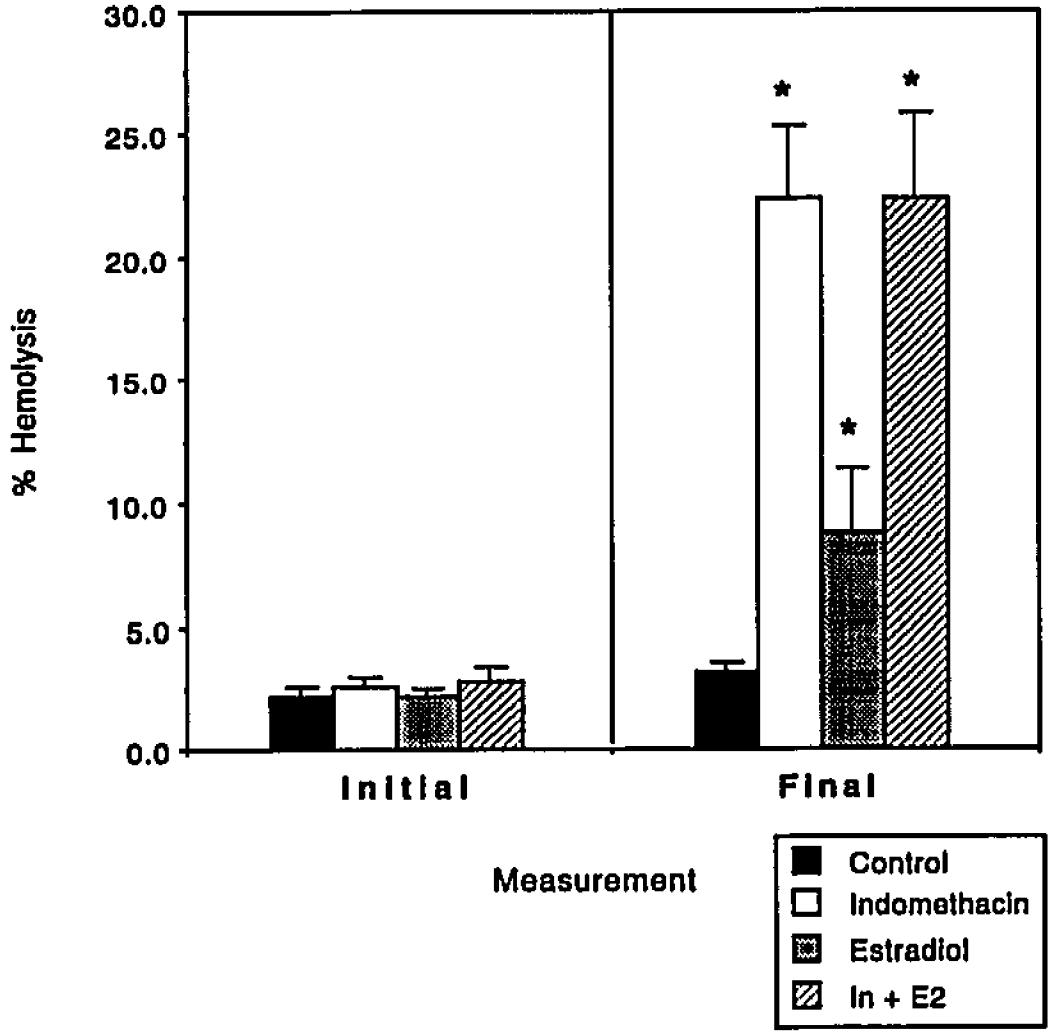


Figure 10. Comparison of mean erythrocyte mechanical fragility hemolysis results (± SEM) for each treatment regimen over time (*Significantly different from final Control value, $p<.05$) ($n = 8$).

Packed Cell Volumes

No significant differences were observed between the mean initial and final PCVs for any of the treatment regimens ($p>.05$), nor were any of the mean final PCVs for the IN, E_2 and IN plus E_2 regimens significantly different from the mean final Control value ($p>.05$). The IN and IN plus E_2-treated sample final microcapillary tube packed cell columns exhibited poorly defined margins with indication of some degree of hemolysis at the cell column-serum interface.

Chapter 4

DISCUSSION

In vivo NZW Rabbit Results

Gross findings.

Administration of IN and E_2-supplementation had no significant effect on spleen weight changes in the OVX or SOVX groups when these compounds were administered alone; however, when they were administered together (IN plus E_2) a significant increase in spleen weight was seen. This suggests that a synergistic interaction is occurring between IN and E_2 in these groups. The lack of significance in mean spleen weights between the low and high dose IN plus E_2-supplemented groups suggests that the interaction is not dose related within this dosage range. The main factor in the interaction may be the constant presence of pharmacologic levels of E_2 since the mean spleen weights in the estrogen-reduced OVX and the normal physiologic estrogen SOVX groups receiving IN were not significantly different from each other, nor were they different from the mean weight of the Intact Control group. Although both IN (Nikcevich *et al.*, 1986; Serushago *et al.*, 1987) and E_2 (Crandall *et al.*, 1980; Sasaki and Ito, 1981) have been associated with occurrence of splenic hematopoiesis which could account for the elevated weights, evaluation of the spleens from the present study showed minimal evidence of splenic hematopoiesis. When hematopoiesis was observed, its' occurrence did not correlate with increased spleen weight.

Histopathology.

Splenic RPM results. The splenic RPM results from this study demonstrate that E_2-supplementation in OVX rabbits stimulates a hyperplastic change in splenic red pulp macrophages. As previously discussed, the mechanism for E_2's stimulatory effect on RPM proliferation is not known. In evaluating the present results, estrogens apparently play a role in maintaining a normal population of RPM in the rabbit since RPM grades for the OVX non-E_2- supplemented groups were, for the most part, significantly lower than the intact and E_2-supplemented groups. As noted earlier, Fluhmann (1928; 1932) observed decreased macrophage numbers in the rabbit endometrium after ovariectomy. Similar ovariectomy-related macrophage effects have also been reported in the guinea pig (Nichol, 1932; 1935) and mouse (Nichol and Vernon-Roberts, 1965). Uebermuth (1933) demonstrated decreased storage ability of macrophages from postmenopausal human females and early work by Antonini *et al.* (1960) linked decreased postmenopausal macrophage activity to an increased incidence of atherosclerosis. These studies suggest that maintenance of physiological estrogen levels may be important in conserving normal monocytic macrophage system function. While decreased numbers of RPM were also observed in the E_2-deficient groups in this study, the macrophages that were present demonstrated a high hemosiderin content indicating that they were very actively involved in erythrophagocytosis. Therefore, it seems from these findings that estrogen may be needed to establish a milieu for splenic RPM proliferation but may not be necessary for normal macrophage function, at least as far as maintaining their capacity for phagocytizing erythrocytes.

The results from this study indicate that E_2's stimulatory effect is sensitive to

the actions of IN. The significant decrease in splenic RPM grade for the SOVX/IN 5.0 group compared to the E_2-supplemented group receiving the same dose of IN makes this assumption plausible. That the RPM grade in the E_2-supplemented group receiving the IN dose of 5.0 mg/kg bw/day in this study was not significantly decreased when compared to the OVX/E_2 Control group while those E_2-supplemented groups in the pilot study receiving IN doses of 10.0 and 13.0 mg/kg bw/day had decreased red pulp macrophage populations, indicates that the IN effect is dose related.

Although the mechanism of interaction of IN with RPM cannot be elucidated from the present experiment, several authors have demonstrated that IN administration can have an effect on cell functions separate from its action on blockade of prostaglandin production. Research by Razin *et al.* (1982) demonstrated decreased binding of IgG-sensitized sheep red blood cells (F_c receptor dependent) by mouse peritoneal macrophages treated *in vitro* with 1 μg/ml IN. They also reported that IN treatment increased plasma membrane binding of cationized ferritin in these cells. While the authors of this study did not completely rule out prostaglandin involvement in their observations, they did make a case for a direct IN-induced reordering of membrane surface charges being at least partially responsible for their results. Indomethacin administration has also been shown to have a direct selective inhibitory effect on the release of lysosomal enzymes (Damas and Bourdon, 1982; Finlay *et al.*, 1975). Other investigators have also reported on IN's and other NSAIDs' direct effects on diverse membrane-associated reactions such as generation of superoxide by the NADPH system of the neutrophil (Blemond *et al.*, 1986), phospholipase C activity in mononuclear cells (Bomalaski *et al.*, 1986), and binding of GTPγS (a stable analog of GTP) to neutrophil membrane preparations (Abramson *et al.*, 1991). Indomethacin was also found

effective in directly inhibiting human platelet membrane-derived and rabbit polymorphonuclear leukocyte PLA_2 (Jesse and Franson, 1979; Kaplan *et al.*, 1978). In both studies the PLA_2 inhibition was dose dependent and occurred at relatively low IN concentrations.

Splenic hemosiderin results. Ovariectomy alone failed to reduce the erythrophagocytic capability of the RPM since the hemosiderin staining level for the OVX Control group was not significantly different from that of the intact Control group. By contrast, the mean scores for the OVX/E_2 Control and OVX/E_2/IN 0.1 were significantly lower than that of the intact Control group. The differences in hemosiderin levels may reflect an adaptation by the macrophages to changes in need for phagocytic capacity, i.e., a smaller population of macrophages adjusts to higher demand for erythrocyte removal by increasing the amount phagocytized, whereas a larger macrophage population proportionally ingests a smaller quantity in response to the same demand. The lack of significance between the hemosiderin staining intensities of the OVX/E_2/IN 5.0 group and its respective control groups may indicate that the 5.0 mg/kg bw/day IN dose is producing increased damage to the erythrocytes resulting in greater phagocytic activity on the part of the RPM. The marked decrease in hematocrit in this group when compared to the OVX/E_2 Control and OVX/E_2/IN 0.1 values also suggests that this IN dose results in increased erythrocyte damage. A thorough search of the literature failed to uncover any research that addressed the issue of changes in phagocytic activity in response to increase or decrease in macrophage population density.

Hematocrits.

Of interest in this investigation was the significant decrease in the 21-day mean hematocrit observed in the OVX Control and OVX/IN 5.0 groups and in all the OVX groups receiving E_2 supplementation. This reduction in hematocrit was more pronounced in the groups that received the high IN dose. Both IN and E_2 have been linked to the induction of aplastic anemia. Aplastic anemia resulting from estrogen administration and characterized by bone marrow hypoplasia has been reported in the dog (Steinberg, 1970) and rat (Tanaka, 1965). Indomethacin administration has been linked to aplastic anemia in humans (Kornberg and Rachmilewitz, 1982). A single case of indomethacin administration-related pure red cell aplasia was reported by Burghuber (1979). Evaluation of the bone marrow sections in the present study failed to detect any significant differences in overall cellularity or numbers of erythroid precursors, indicating that the observed hematocrit changes are not due to decreases in red blood cell production. While the hematocrit changes in the OVX Control group could be attributed to a response to surgical trauma, in the other groups a likely explanation for the decrease may be related to direct action of both IN and E_2 on erythrocyte plasma membranes. Both of these compounds have been shown to produce changes in erythrocyte plasma membranes. Red blood cells from women who used ethinyl estradiol-based birth control pills were found to have reduced membrane deformabilities (Heilmann *et al.*, 1981). Estrogenic compounds were also found to block the *in vitro* uptake of monosaccharides by human erythrocytes as well as decreasing their membrane integrity (Ingermann, 1989). Addition of IN to washed canine RBCs resulted in increased membrane stability as determined by increased resistance to heat-induced hemolysis (Brown *et al.*, 1967). Indomethacin-related RBC hemolysis has been reported in animal studies. Giglio and Bozzini (1982)

reported a transient decrease in hematocrit along with increased hemolysis in female mice receiving a single 400 μg dose of IN. Indomethacin has also been implicated, even if anecdotally, in the autoimmune form of hemolytic anemia in humans (Epner, 1994).

The splenic RPM play a major role in the removal of aged and altered erythrocytes from the circulation (Auger and Ross, 1992) and have been shown to be able to detect RBCs with only slight plasma membrane damage (Harris *et al.*, 1957). Drug-induced red cell membrane damage of a minor nature would first be detected by splenic RPM that would remove these cells from the circulation. Continued damage to increasing numbers of erythrocytes would further activate other splenic RPM and result in an increased cell population in the splenic cords, producing the increase in RPM grade observed in this study. Kay (1981) demonstrated an IgG autoantibody-binding antigen on senescent and damaged mammalian erythrocytes and somatic cells that she feels is responsible for their removal from the circulation by macrophages. Other investigators have shown in *in vitro* studies that under certain pathologic conditions (e.g., favism, *P. falciparum* infection and sickle cell anemia) a complement receptor (CR)-mediated mechanism may be responsible for elimination of damaged erythrocytes (Arese *et al.*, 1990). While a CR-mediated mechanism for removal of damaged erythrocytes cannot be ruled out, work by Buckley *et al.* (1987) has demonstrated that over 75% of splenic RPM stain positively for F_cR (responsible for binding IgG coated particles) while less than 10% of these cells are positive for complement factor CR3 receptors (responsible for the phagocytosis of complement opsonized particles). In support of a F_cR-mediated mechanism of splenic erythrocyte removal, Schroit *et al.* (1985) have shown that CR-mediated removal of RBCs occurs primarily by action of Kupffer cells in the liver.

Although an immune-mediated clearance mechanism may be responsible for the

removal of most erythrocytes from circulation, a non-immune mechanism has also been described. Conner *et al.* (1994), in studies carried out in antibody-deficient SCID mice, observed that aged red blood cells were removed from the circulation via a mechanism that was independent of antibody involvement. They reported that the erythrocyte removal rate by this non-immune mechanism was comparable to the clearance rate in immunocompetent BALB/c mice. The possible importance of this non-immune removal pathway in normal animals was not addressed, nor was its' role in the removal of damaged erythrocytes discussed. Sambrano *et al.* (1994) have also described non-antibody-dependent scavenger receptor mediated phagocytosis of oxidatively damaged erythrocytes by mouse peritoneal macrophages.

Conclusion

The results of the *in vivo* study indicate that a complex interaction is occurring with coadministration of E_2 and IN. There is an apparent direct effect of both drugs on the splenic RPM, with E_2 producing a stimulatory effect and IN directly acting to partially inhibit the effect of normal physiological levels of estrogen. Estradiol administration appears primarily to mediate an increase in macrophage population and not to affect macrophage erythrophagocytic capacity. Simultaneously, both drugs may be acting on erythrocyte plasma membranes to change their fragility characteristics, prompting increased stimulation of the splenic RPM to actively remove these damaged erythrocytes from the circulation, thereby lowering hematocrit.

In vitro NZW Rabbit Erythrocyte Fragility Results

The results from the *in vitro* study indicate that IN treatment increases both osmotic and mechanical fragility of rabbit erythrocytes. The osmotic fragility findings

differ from the thermal hemolysis results of Kalbhen *et al.* (1970) who found that IN added to washed human erythrocytes stabilized the membranes against heat-induced hemolysis over a range of concentrations (1×10^{-5} to 5×10^{-4} M). Tanaka *et al.* (1973) have also reported temperature dependent changes in hemolysis characteristics of washed rat erythrocytes in response to treatment with IN and other NSAIDs. In their work they noted that addition of these compounds inhibited hypotonic erythrocyte lysis at 0°C while promoting lysis at 37°C. Temperature may be an independent variable in its influence on hemolysis when compared to changes induced by osmotic stress since previous experiments conducted under similar conditions as the present work, except for incubation of the samples at 4°C, resulted in decreased osmotic fragility of the IN-treated rabbit erythrocytes (data not shown).

The erythrocyte fragility results of IN treatment observed in this study suggest that not only does it have an influence on the capacity of the erythrocyte cell membrane to accommodate to osmotic stress but that it also has an effect on the ability of the cytoskeletal structure to respond to mechanical stress. The effect of IN on erythrocyte mechanical fragility properties appear dose related since Mizushima *et al.* (1970) demonstrated that doses of IN which were more than 10 times greater than those used in my study protected canine erythrocytes against mechanical stress.

The mechanism or mechanisms of erythrocyte interaction with IN and other NSAIDs have yet to be conclusively determined although some authors have reported that they bind to the outer erythrocyte membrane in a charge-related manner (Sheetz and Singer, 1974; Aki and Yamamoto, 1990). As a class, NSAIDs are planar, anionic compounds that partition into lipid environments such as the cell membrane bilayer. As discussed above, several investigators have reported that this ability results in their involvement in inhibition of diverse non-prostaglandin mediated, membrane-associated

reactions (Biemond *et al.*, 1986; Bomalaski *et al.*, 1986; Abramson *et al.*, 1991). Another possible mechanism for IN's effect on the erythrocyte membrane may be through direct binding to the band 3 anion transporter protein. This glycoprotein is the major transmembrane protein found in the erythrocyte and is essential in the CO_2 transport system because of its transmembrane exchange of Cl^- and HCO_3^- (Passow, 1986). Although anion transport appears the major function of the band 3 protein it also may be involved in red blood cell hypotonic hemolysis (Sato *et al.*, 1993) and, because of its connection to spectrin via its hinged cytosolic domain, also appears to serve as a flexible membrane anchor for the erythrocyte actin-spectrin cytoskeleton (Wang, 1994). Of interest in investigating the interaction of IN and other NSAIDs with the erythrocyte is the structure of the band 3 protein extracytoplasmic water entrance channel. This channel is lined by cationic amino acids consisting primarily of lysine residues (Widdas and Baker, 1993). Indirect evidence of interaction of IN with lysine has been provided by Mizushima and Sakai (1969) who demonstrated that the lysine ε-amino group binding compound 2,4,6-trinitrobenzene sulfonic acid inhibited the membrane stabilizing effect of IN against heat-induced hemolysis of canine erythrocytes. Direct evidence of IN involvement in cellular membrane anion exchange has been shown by Tønnessen *et al.* (1989) who reported that IN inhibited transmembrane exchange of Cl^- and HCO_3^- in cultured Vero cells via a non-prostaglandin-mediated pathway. Possible membrane surface interaction of anionic NSAIDs is also supported by the work of Aki and Yamamoto (1990), who used flow microcalorimetry techniques measuring changes in enthalpy and entropy to conclude that these drugs bind to erythrocyte surface membrane proteins. Observations from my research that both osmotic and mechanical fragility properties of the rabbit erythrocytes were affected by IN indicate that the

potential exists for IN's involvement with the band 3 protein.

The addition of E_2 to rabbit erythrocytes produced changes only in erythrocyte MF characteristics. The increased MF observed may indicate that, at least under these experimental conditions, E_2's effect is primarily through hindrance of the ability of the cytoskeleton to accommodate to changes in mechanical stress. Other researchers have reported changes in mechanical characteristics of erythrocytes obtained from women who used oral contraceptives (Heilmann *et al.*, 1981; Oski *et al.* 1972). In both these studies the investigators observed that erythrocytes had a decreased ability to deform, indicating that they would be more labile under conditions of mechanical stress. While it is tempting to draw correlations between the present *in vitro* study and the studies of Heilmann and Oski, it should be noted that other research has shown that estrogen administration *in vivo*, either as estradiol or as ethinyl estradiol, can produce changes in erythrocyte membrane lipid composition that may alter their fragility characteristics (Cho *et al.*, 1988; Le-Petit-Thevenin *et al.*, 1986; Fehily *et al.*, 1982). While no E_2-related effect on OF characteristics was observed in the present study, Florence and Rahman in their *in vitro* research (1972) have demonstrated an E_2-protective effect of erythrocytes against hypotonic hemolysis. In this study the authors added approximately 35 μg of E_2 in an aqueous solution to 1 ml of washed erythrocytes diluted by a factor of at least 1000 to 1. In the present work a low concentration of water-soluble E_2 (500 pg/ml) was added to whole blood with erythrocyte PCVs ranging from 25% to 34%. Given the results of the current research and that of Florence and Rahman, it would appear that the effect of E_2 on erythrocyte OF is dose related.

Estradiol's mechanism of erythrocyte interaction has yet to be fully elaborated. Brinkman *et al.* (1972a) observed that steroids in general move rapidly across

erythrocyte membranes apparently by the mechanism of simple diffusion; however, other research has shown that binding will occur to isolated membrane proteins (Brinkmann and Van der Molen, 1972b; DeVenuto *et al.*, 1969). Brinkmann and Van der Molen (1972b) evaluated human erythrocyte membrane protein binding of E_2 and other steroids *in vitro* and reported that binding was nonsaturable and decreased with decreasing polarity of the steroid. These findings led them to conclude that steroid-membrane protein interactions are nonspecific in nature. Given the similarity in structure of steroids to the cholesterol molecule and their ability to readily partition from aqueous to lipid environments, it was felt that steroids may interact with membranes through direct intercalation into the lipid bilayer (Willmer, 1961). Subsequent *in vitro* research, however, has shown that many steroids, including E_2, may react primarily with the hydrated polar head groups of membrane phospholipids and orient parallel to the water-lipid interface (Cleary and Zatz, 1977; Khalat *et al.*, 1975; Florence and Rahman, 1972). Work using calorimetric and Raman spectroscopic techniques to assess the interaction of anesthetic and nonanesthetic steroids with dipalmitoylphosphatidyl-choline liposomes suggests that a membrane lipid-mediated mechanism is involved in the action of these substances (O'Leary *et al.*, 1984). Recent work by Koefoed and Brahm (1994) has shown that sex hormones added to human erythrocytes can pass through the cell membrane and bind to cytoplasmic components but are predominantly found bound to membrane-associated structures. Given the different explanations for the mechanism of steroid and erythrocyte interaction, it is difficult to draw definitive conclusions on E_2's role in the alteration of membrane fragility characteristics. If the membrane lipid-mediated mechanism of interaction is correct, then it would be easy to hypothesize that partitioning of E_2 into the membrane

bilayer may produce the changes in mechanical fragility that we observed. McPherson *et al.* (1992) have reported that band 3 protein's integral domain undergoes rotational diffusion within the erythrocyte membrane under normal physiological conditions, hindrance of this diffusion by intercalation of E_2, or other steroid, into the lipid bilayer may compromise the ability of the erythrocyte to deform in response to mechanical stress.

The combination of IN plus E_2 added to rabbit erythrocytes failed to produce a significant change in fragility characteristics when compared to IN addition alone. This apparent lack of an additive, synergistic or diminutional effect may indicate that the two drugs vary either in their degree of membrane involvement or have different loci or mechanisms of interaction.

Conclusions.

When IN and E_2 alone were added to rabbit whole blood at 39.5°C, both drugs produced increases in mechanical fragility after 4 hours. Only IN had an effect on osmotic fragility, producing a decrease in the ability of the erythrocytes to respond to changes in osmotic stress. The degree of increased fragility was great enough to significantly increase the values of p1 (50% hemolysis point) and p2 (hemolysis dispersion) but not sufficient to make a significant change in PCV. Changes in these factors appear to be more critical indicators of fragility differences in rabbit erythrocytes than is the PCV. Combination of the two drugs failed to produce results that were significantly different from the mechanical fragility results for IN alone, indicating a lack of additive, synergistic or antagonistic interaction of the two compounds.

Chapter 5

SUMMARY CONCLUSIONS AND FUTURE PROSPECTS

Summary Conclusions

In vivo NZW Rabbit Findings Summary

- Ovariectomy reduced splenic red pulp macrophage grade but did not decrease erythrophagocytic activity.
- Estradiol supplementation increased splenic red pulp macrophage grade of ovariectomized animals.
- Indomethacin decreased red pulp macrophage grade in SOVX/IN 5.0 groups versus the OVX/E_2/IN 5.0 group.
- Indomethacin and estradiol administration decreased rabbit hematocrits

In vitro NZW Rabbit Erythrocyte Fragility Findings Summary

- Indomethacin increased rabbit erythrocyte mechanical and osmotic fragility.
- Estradiol increased rabbit erythrocyte mechanical fragility.
- No interactive effect of indomethacin and estradiol on rabbit erythrocyte fragility characteristics was observed.

The results of the *in vivo* experiment confirm the hypothesis of a dose-related IN inhibition of E_2 stimulation of RPM activity. They also demonstrate that the effect of E_2 on the RPM is primarily that of increasing the number of macrophages rather than affecting their phagocytic capacities. Further, normal physiological levels of E_2 protect against the high IN dose-induced decreases in mean hematocrit observed in the OVX and OVX/E_2 groups. The *in vitro* results support the hypothesis that IN-induced erythrocyte damage contributes to the decreases observed in

the *in vivo* hematocrits of those OVX groups receiving the high IN dose.

The *in vivo* findings agree with other researcher's reports on the stimulatory effects of estradiol on cells of the monocyte macrophage system. The clinical significance of this effect on splenic macrophages has never been addressed and it is possible that if it does occur in humans it may go undetected. Given the cyclic nature of estrogen production under normal physiological conditions, changes in splenic macrophage numbers in response to estrogen level fluctuations may be transient. The exact nature of estradiol's stimulatory activity is still a matter of conjecture although, given the literature findings, it is likely mediated by receptor interaction. The exact location of the splenic red pulp macrophage estradiol binding site still remains to be determined. Indomethacin' s capacity to partially reduce the red pulp macrophage grade in the SOVX/IN 5.0 animals in relation to the OVX/E_2/IN 5.0 animals may be related to its ability to interfere with E_2's stimulatory effect on these macrophages rather than blockade of erythrophagocytosis. This is supported by the fact that there was no significant difference in mean hemosiderin grade between that of the SOVX/IN 5.0 group and its treatment regimen control group. Whether indomethacin's inhibitory actions on the splenic macrophages involves the same mechanism as does estradiol's stimulatory activity is not known.

The effect of both indomethacin and estradiol on the *in vivo* hematocrits indicates that there may be a direct effect of these drugs on the erythrocyte. The erythrocyte fragility responses to indomethacin observed in the *in vitro* study have not been reported to occur *in vivo* although one group of researchers, evaluating the effects of several NSAIDs on changes in membrane structure using electron spin resonance measurements of human male and female erythrocytes, did report that ingestion of indomethacin (at a

level of 50 mg) slightly changed the resonance characteristics of erythrocytes from both sexes (Mazorow *et al.*, 1985). These authors also investigated the *in vitro* effect of indomethacin (at 2 μg/ml) on washed erythrocytes, noting no significant changes in membrane structure. Comparing the*in vitro* results obtained with the IN dose used by these investigators to the results from the present study using a IN dose of 9.6 μg/ml indicates that a dose-response relationship exists in IN's effect on erythrocyte membrane properties.

Future Prospects

Additional *in vivo* and *in vitro* (using either isolated macrophages or a macrophage cell line) research needs to be done to clarify the mechanism of estradiol interaction with the splenic red pulp macrophages. This would consist of immunohistochemical procedures to determine where on the cell estradiol binds (i.e., cell membrane versus nuclear binding) as well as its binding density. Changes in cytokine levels (e.g., IL-1, IL-3, etc.) should be monitored in the *in vivo* and *in vitro* studies. Erythropoietin changes should also be measured in the *in vivo* investigations. Further *in vivo* work should be done to confirm the dose-response relationship of indomethacin's effect on the estradiol-induced splenic red pulp macrophage changes, as well as the nature of the interaction. This study would also assess the *in vivo* effect of indomethacin and estradiol on the erythrocyte membranes in terms of both fragility and membrane composition changes. The effect of IN and E_2 on erythrocyte life-span should also be assessed to determine whether erythrocytes in animals receiving one or both of these drugs have shorter life spans and therefore, higher rates of removal.

Two early *in vitro*, whole blood experiments showed that estradiol alone had an influence on both mechanical and osmotic erythrocyte fragility, as well as changing the

Indomethacin-induced fragility responses of the erythrocytes when administered along with that drug. Another set of *in vitro* experiments using washed erythrocytes should be conducted to address the possibility of an estradiol dose-response effect on both fragility parameters. Further *in vitro* studies should also be done to determine the time course of IN and E_2 erythrocyte binding.

A second area of research would be to evaluate the effects of other NSAIDs in the same research paradigms. These studies would determine whether the *in vivo* and *in vitro* results observed using indomethacin are common to the NSAIDs as a class.

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APPENDIX

Spleen Weights ANOVA Results

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
TREATMENT G...	9	9.475	1.053	4.562	.0008
Residual	29	6.663	.230		

Dependent: Spleen Wgt

Means Table
Effect: TREATMENT GROUP
Dependent: Spleen Wgt

	Count	Mean	Std. Dev.	Std. Error
1	4	1.165	.158	.079
2	4	.977	.277	.138
3	4	1.225	.279	.139
4	4	1.240	.441	.220
5	4	1.367	.535	.267
6	4	1.128	.361	.180
7	4	1.450	.222	.111
8	4	2.472	.928	.464
9	4	2.357	.688	.344
10	3	1.433	.266	.153

Duncan New Multiple Range
Effect: TREATMENT GROUP
Dependent: Spleen Wgt
Significance level: .05

	Vs.	Diff.	Crit. diff.	
2	8	.150	.705	
	1	.188	.741	
	3	.248	.761	
	4	.263	.780	
	5	.390	.793	
	10	.456	.803	
	7	.473	.810	
	9	1.380	.816	S
	6	1.495	.821	S
6	1	.038	.705	
	3	.097	.741	
	4	.112	.761	
	5	.240	.780	
	10	.306	.793	
	7	.322	.803	
	9	1.230	.810	S
	8	1.345	.816	S
1	3	.060	.705	
	4	.075	.741	
	5	.202	.761	
	10	.268	.780	
	7	.285	.793	
	9	1.192	.803	S
	8	1.307	.810	S
3	4	.015	.705	
	5	.142	.741	
	10	.208	.761	
	7	.225	.780	
	9	1.132	.793	S
	8	1.247	.803	S
4	5	.127	.705	
	10	.193	.741	
	7	.210	.761	
	9	1.117	.780	S
	8	1.232	.793	S
5	10	.066	.705	
	7	.083	.741	
	9	.990	.761	S
	8	1.105	.780	S
10	7	.017	.705	
	9	.924	.741	S
	8	1.039	.761	S
7	9	.907	.705	S
	8	1.022	.741	S
9	8	.115	.705	

S = Significantly different at this level.

RPM - ANOVA Results

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
TREATMENT G...	9	19.942	2.216	4.080	.0018
Residual	29	15.750	.543		

Dependent: RE Hyperplasia

Means Table
Effect: TREATMENT GROUP
Dependent: RE Hyperplasia

	Count	Mean	Std. Dev.	Std. Error
1	4	1.750	.500	.250
2	4	1.250	.500	.250
3	4	1.750	1.258	.629
4	4	2.750	.500	.250
5	4	2.500	.577	.289
6	4	2.000	.816	.408
7	4	3.250	.500	.250
8	4	3.000	.816	.408
9	4	3.500	.577	.289
10	3	3.000	1.000	.577

Duncan New Multiple Range
Effect: TREATMENT GROUP
Dependent: RE Hyperplasia
Significance level: .05

	Vs.	Diff.	Crit. diff.	
2	1	.500	1.084	
	3	.500	1.139	
	8	.750	1.171	
	5	1.250	1.199	S
	4	1.500	1.219	S
	10	1.750	1.234	S
	6	1.750	1.245	S
	7	2.000	1.255	S
	9	2.250	1.262	S
1	3	0.000	1.084	
	8	.250	1.139	
	5	.750	1.171	
	4	1.000	1.199	
	10	1.250	1.219	S
	8	1.250	1.234	S
	7	1.500	1.245	S
	9	1.750	1.255	S
3	8	.250	1.084	
	5	.750	1.139	
	4	1.000	1.171	
	10	1.250	1.199	S
	8	1.250	1.219	S
	7	1.500	1.234	S
	9	1.750	1.245	S
6	5	.500	1.084	
	4	.750	1.139	
	10	1.000	1.171	
	8	1.000	1.199	
	7	1.250	1.219	S
	9	1.500	1.234	S
5	4	.250	1.084	
	10	.500	1.139	
	8	.500	1.171	
	7	.750	1.199	
	9	1.000	1.219	
4	10	.250	1.084	
	8	.250	1.139	
	7	.500	1.171	
	9	.750	1.199	
10	8	0.000	1.084	
	7	.250	1.139	
	9	.500	1.171	
8	7	.250	1.084	
	9	.500	1.139	
7	9	.250	1.084	

S = Significantly different at this level.

Hemosiderin ANOVA Results

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
TREATMENT G...	9	26.827	2.981	2.476	.0311
Residual	29	34.917	1.204		

Dependent: HEMO. - IRON

Means Table
Effect: TREATMENT GROUP
Dependent: HEMO. - IRON

	Count	Mean	Std. Dev.	Std. Error
1	4	2.750	1.500	.750
2	4	2.250	.957	.479
3	4	3.250	.500	.250
4	4	2.750	.957	.479
5	4	2.000	.816	.408
6	4	2.250	1.258	.629
7	4	1.250	.957	.479
8	4	.500	.577	.289
9	4	1.750	1.708	.854
10	3	3.333	1.155	.667

Duncan New Multiple Range
Effect: TREATMENT GROUP
Dependent: HEMO. - IRON
Significance level: .05

	Vs.	Diff.	Crit. diff.	
8	7	.750	1.615	
	9	1.250	1.695	
	5	1.500	1.743	
	2	1.750	1.785	
	6	1.750	1.815	
	4	2.250	1.838	S
	1	2.250	1.854	S
	3	2.750	1.868	S
	10	2.833	1.879	S
7	9	.500	1.615	
	5	.750	1.695	
	2	1.000	1.743	
	6	1.000	1.785	
	4	1.500	1.815	
	1	1.500	1.838	
	3	2.000	1.854	S
	10	2.083	1.868	S
9	5	.250	1.615	
	2	.500	1.695	
	6	.500	1.743	
	4	1.000	1.785	
	1	1.000	1.815	
	3	1.500	1.838	
	10	1.583	1.854	
5	2	.250	1.615	
	6	.250	1.695	
	4	.750	1.743	
	1	.750	1.785	
	3	1.250	1.815	
	10	1.333	1.838	
2	6	0.000	1.615	
	4	.500	1.695	
	1	.500	1.743	
	3	1.000	1.785	
	10	1.083	1.815	
6	4	.500	1.615	
	1	.500	1.695	
	3	1.000	1.743	
	10	1.083	1.785	
4	1	0.000	1.615	
	3	.500	1.695	
	10	.583	1.743	
1	3	.500	1.615	
	10	.583	1.695	
3	10	.083	1.615	

S = Significantly different at this level.

Paired t-Tests for Pretreatment and 21-day Hematocrits

OVX Control

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	1.75	3.6556	.0354

OVX/IN 0.1

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	1.375	1.2026	.3154

OVX/IN 5.0

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	2.625	2.4579	.091

SOVX Control

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	-1.625	-1.4945	.2319

SOVX/IN 0.1

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	2.625	1.4434	.2446

SOVX/IN 5.0

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	.875	.7534	.506

OVX/E2 Control

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	2.75	2.7218	.0724

OVX/E2/IN 0.1

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	5.125	4.0012	.028

OVX/E2/IN 5.0

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
3	7	3.2404	.0478

Control

Paired t-Test X 1 : Pre Hemcrit Y 1 : Post Hemcrit

DF:	Mean X - Y:	Paired t value:	Prob. (2-tail):
2	.5	.2774	.8075

Serum estradiol levels - ANOVA Results

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
TREATMENT G...	5	662851.313	132570.263	5.721	.0025
Residual	18	417132.380	23174.021		

Dependent: E2 Levels 1

Means Table
Effect: TREATMENT GROUP
Dependent: E2 Levels 1

	Count	Mean	Std. Dev.	Std. Error
1	4	108.950	16.823	8.412
2	4	114.825	38.100	19.050
3	4	158.275	67.642	33.821
7	4	504.700	179.719	89.859
8	4	394.975	197.887	98.943
9	4	456.675	247.540	123.770

Duncan New Multiple Range
Effect: TREATMENT GROUP
Dependent: E2 Levels 1
Significance level: .05

	V...	Diff.	Crit. diff.	
1	2	5.875	226.062	
	3	49.325	237.479	
	8	286.025	244.329	S
	9	347.725	248.896	S
	7	395.750	252.702	S
2	3	43.450	226.062	
	8	280.150	237.479	S
	9	341.850	244.329	S
	7	389.875	248.896	S
3	8	236.700	226.062	S
	9	298.400	237.479	S
	7	346.425	244.329	S
8	9	61.700	226.062	
	7	109.725	237.479	
9	7	48.025	226.062	

S = Significantly different at this level.

P1 2-way ANOVA - Treatment * Interval

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	3	.006	.002	4.120	.0104
Interval	1	.027	.027	53.140	.0001
Treatment * Int...	3	.007	.002	4.271	.0087
Residual	56	.029	.001		

Dependent: P1

Least Squares Means Table
Effect: Treatment * Interval
Dependent: P1

	Vs.	Diff.	Std. Error	t-Test	P-Value
Control, Initial	Control, Final	-.020	.011	-1.756	.0845
	Indomethacin, Initial	.001	.011	.114	.9097
	Indomethacin, Final	-.060	.011	-5.346	.0001
	Estradiol, Initial	.001	.011	.107	.9150
	Estradiol, Final	-.021	.011	-1.850	.0696
	I + E, Initial	.001	.011	.054	.9570
	I + E, Final	-.060	.011	-5.352	.0001
Control, Final	Indomethacin, Initial	.021	.011	1.870	.0667
	Indomethacin, Final	-.041	.011	-3.590	.0007
	Estradiol, Initial	.021	.011	1.863	.0677
	Estradiol, Final	-.001	.011	-.094	.9254
	I + E, Initial	.020	.011	1.810	.0756
	I + E, Final	-.041	.011	-3.596	.0007
Indomethacin, Initial	Indomethacin, Final	-.062	.011	-5.460	.0001
	Estradiol, Initial	-7.500E-5	.011	-.007	.9947
	Estradiol, Final	-.022	.011	-1.964	.0545
	I + E, Initial	-.001	.011	-.060	.9526
	I + E, Final	-.062	.011	-5.466	.0001
Indomethacin, Final	Estradiol, Initial	.062	.011	5.453	.0001
	Estradiol, Final	.040	.011	3.496	.0009
	I + E, Initial	.061	.011	5.400	.0001
	I + E, Final	-7.500E-5	.011	-.007	.9947
Estradiol, Initial	Estradiol, Final	-.022	.011	-1.957	.0553
	I + E, Initial	-.001	.011	-.053	.9579
	I + E, Final	-.062	.011	-5.460	.0001
Estradiol, Final	I + E, Initial	.022	.011	1.904	.0620
	I + E, Final	-.040	.011	-3.502	.0009
I + E, Initial	I + E, Final	-.061	.011	-5.406	.0001

P2 2-Way ANOVA Results

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	3	.001	3.543E-4	.843	.4762
Interval	1	.013	.013	30.025	.0001
Treatment * Int...	3	.001	3.810E-4	.906	.4439
Residual	56	.024	4.204E-4		

Dependent: P2

Least Squares Means Table
Effect: Treatment * Interval
Dependent: P2

	Vs.	Diff.	Std. Error	t-Test	P-Value
Control, Initial	Control, Final	-.019	.010	-1.874	.0661
	Indomethacin, Initial	-.002	.010	-.167	.8679
	Indomethacin, Final	-.037	.010	-3.646	.0006
	Estradiol, Initial	-.003	.010	-.263	.7932
	Estradiol, Final	-.023	.010	-2.225	.0301
	I + E, Initial	2.625E-4	.010	.026	.9797
	I + E, Final	-.037	.010	-3.619	.0006
Control, Final	Indomethacin, Initial	.018	.010	1.707	.0934
	Indomethacin, Final	-.018	.010	-1.772	.0819
	Estradiol, Initial	.017	.010	1.611	.1129
	Estradiol, Final	-.004	.010	-.351	.7268
	I + E, Initial	.019	.010	1.900	.0628
	I + E, Final	-.018	.010	-1.745	.0865
Indomethacin, Initial	Indomethacin, Final	-.036	.010	-3.479	.0010
	Estradiol, Initial	-.001	.010	-.096	.9236
	Estradiol, Final	-.021	.010	-2.058	.0442
	I + E, Initial	.002	.010	.193	.8479
	I + E, Final	-.035	.010	-3.452	.0011
Indomethacin, Final	Estradiol, Initial	.035	.010	3.382	.0013
	Estradiol, Final	.015	.010	1.420	.1610
	I + E, Initial	.038	.010	3.671	.0005
	I + E, Final	2.750E-4	.010	.027	.9787
Estradiol, Initial	Estradiol, Final	-.020	.010	-1.962	.0548
	I + E, Initial	.003	.010	.289	.7737
	I + E, Final	-.034	.010	-3.355	.0014
Estradiol, Final	I + E, Initial	.023	.010	2.251	.0283
	I + E, Final	-.014	.010	-1.394	.1689
I + E, Initial	I + E, Final	-.037	.010	-3.644	.0006

Mechanical Fragility - One-way ANOVA

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Treatment	3	6809.326	2269.776	13.927	.0001
Residual	92	14993.702	162.975		

Dependent: Four

Tukey-Kramer
Effect: Treatment
Dependent: Four
Significance level: .05

	Vs.	Diff.	Crit. diff.	
Control	Estradiol	10.653	9.676	S
	Indomethacin	19.133	9.676	S
	In + E2	19.158	9.676	S
Estradiol	Indomethacin	13.612	9.676	S
	In + E2	13.638	9.676	S
Indomethacin	In + E2	.025	9.676	

S = Significantly different at this level.

VITAE

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EDUCATION:

5/69	B.S.	Biology; Virginia Polytechnic Institute and State University; Blacksburg, Virginia.
6/79	M.S.	Pathobiology; University of Connecticut; Storrs, Connecticut.
5/95	Ph.D.	Biomedical Sciences (Pharmacology); James H. Quillen College of Medicine, East Tennessee State University; Johnson City, Tennessee.

EMPLOYMENT HISTORY:

12/83 - present	Research Specialist; Department of Pathology, James H. Quillen College of Medicine; East Tennessee State University; Johnson City, Tennessee.
10/81 - 3/83	Project Controller and Document Researcher; Bioassay Systems Corporation; Woburn, Massachusetts.
5/80 - 10/81	Screening Histopathologist and Technical Manager; Bioassay Systems Corporation; Old Saybrook, Connecticut and Woburn, Massachusetts.

TEACHING EXPERIENCE:

4/91 - present	Lecturer in Medical Pharmacology; "Antihyperlipidemic Drugs."
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PUBLICATIONS:

Original Articles:

1. Shurbaji, MS, Kuhajda, FP, Pasternack, GR, and Thurmond, TS (1992). Expression of Oncogenic Antigen 519 (OA-519) in prostate cancer is a potential prognostic indicator. *Am. J. Clin. Pathol.* 97:689-91.

2. Shurbaji, MS, Brooks, SK, and Thurmond, TS (1993). Proliferating cell nuclear antigen (PCNA) immunoreactivity in cervical intraepithelial neoplasia. *Am. J. Clin. Pathol.* 100:22-6.

3. Shurbaji, MS, Kalbfleisch, JH, and Thurmond, TS (1994). Immunohistochemical detection of p53 protein as a prognostic indicator in prostate cancer. *Hum. Pathol.* 26:106-9

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1. Zollars, PR, Thurmond, TS, Musil, G, and Coogan, PS (1985). Splenomegaly resulting from short-term estrogen administration in rabbits. *Fed. Proc.* 44:845.

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10. Shurbaji, MS, Kalbfleisch, JH, and Thurmond, TS (1994). Immunohistologic detection of p53 protein and prognosis of prostate cancer. *Mod. Pathol.* 7:83A.

11. Ferslew, KE, Orcutt, RH, and Thurmond, TS (1995). *In vitro* effects of estradiol (E_2) and indomethacin (IN) on rabbit erythrocyte fragility (EF) characteristics. *Toxicologist* 15:221.

12. Orcutt, RH, Thurmond TS, and Ferslew, KE (1995). Mathematical modeling of the osmotic fragility of rabbit red blood cells. *Toxicologist* 15:222.

13. Thurmond, TS, Ferslew, KE, McCracken, MD, and Coogan, PS (1995). Toxicopathology of indomethacin in the spleen of ovariectomized, New Zealand White (NZW) rabbits. *Toxicologist* 15:227.

MANUSCRIPTS SUBMITTED FOR PUBLICATION:

1. Thurmond, TS, Ferslew, KE, Orcutt, RH, and Coogan, PS. In vitro effects of estradiol and indomethacin on rabbit erythrocyte fragility characteristics.

2. Shurbaji, MS, Kalbfleisch, JH, and Thurmond, TS. Immunohistochemical detection of a fatty acid synthase (OA 519) as a predictor of progression in prostate cancer.

3. Thurmond, TS, Ferslew, KE, McCracken, MD, and Coogan, PS. The effect of indomethacin administration on the splenic changes induced by estradiol supplementation in ovariectomized New Zealand White rabbits.