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MOUSE EMBRYO DEVELOPMENT IN THE PRESENCE OF CAPSAICIN

by

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BA May 1984, University of Virginia

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ABSTRACT

MOUSE EMBRYO DEVELOPMENT IN THE PRESENCE OF CAPSAICIN.

Carlos S. Villar-Gosalvez
Old Dominion University, 1998
Director: Dr. R. James Swanson

Capsaicin is the pungent agent found in hot peppers of the Capsicum genus. It is a potent neurotoxin that stimulates the degranulation and degeneration of C-afferent neurons. Capsaicin is widely used as a food condiment and medicine. Human exposure of capsaicin can exceed levels shown to be neurotoxic in laboratory animals. Additionally, capsaicin can cross the blood/placenta barrier and affect an embryo in utero. In order to assay the potential for toxicity to human embryos, mouse embryos were exposed to capsaicin and the effect of the capsaicin on embryo development was measured. Embryos were co-cultured in Krebs medium with 1% ethanol and from 1 to 3mM capsaicin. The higher levels of capsaicin significantly inhibited embryo development. Post implantation fetuses were treated in the dam with 0.3, 0.6, 1.5, and 3µmoles capsaicin, and tested for developmental defects. No significant differences were found between the capsaicin treated fetuses and the control fetuses. Female mice were exposed to 3µmoles capsaicin sub-cutaneously, by mouth, and topically. Levels of capsaicin in their blood serum were measured by high-pressure liquid chromatography. No significant levels of capsaicin were detected. It was concluded that any deleterious effect of

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capsaicin on embryo or fetal development depends on very high dosages and that these levels are unlikely to be encountered in the blood. Also, capsaicin had no deleterious effect on cartilage, bone, or limb development in mice.

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This Dissertation is dedicated to my family:

my parents,

Carlos Villar-Palasi and Amparo Gosalvez-Sobrino,

who have helped and inspired me throughout

my academic career,

to my brothers Victor and Juan, my Sister Amparo, my Sister-In-Law Victoria,

my Nieces Maria and Victoria

and other members of my family whom I hold in my heart, and

who have been an immense source of help, love, and support

during the times when the HPLC leaked, or the mice did not mate,

or the computer crashed.

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INTRODUCTION

HOT PEPPERS, SEXUAL REPRODUCTION, AND THE ROLE OF GENITAL INNERVATION

The winner of a local hot pepper eating contest in Norfolk, Virginia, ingested 13 Habanero peppers weighing about 10 grams each. This means that she received an estimated dose of 50 milligrams per kilogram of capsaicin. Fifty milligrams per kilogram have been shown to cause total degeneration of the C-afferent fibers of the sensory nervous system in laboratory mice. This person may have caused herself permanent neurological and reproductive damage. Capsaicin, (8-methyl-N-vanillyl-6-nonenamide), is one of a small group of very pungent compounds found in hot peppers of the *Capsicum* genus (Govindarajan, and Sathyanarayana, 1991). These peppers are also known as Chilis, Jalapeños, Paprika, Guindillas, Cayenne, and Red pepper. They are a very commonly encountered food and condiment, are used in medicines and, in the form of pepper spray, in personal defense preparations (Dasgupta and Fowler, 1997). Chronic and acute exposure to capsaicin in highly concentrated form is commonplace, and a host of pharmacological effects has been reported as a result (Buck and Burks, 1986). In the 1940's and 50's, a Hungarian scientist named Nicolas Jancsó began studying the neurotoxic and inflammatory properties of capsaicin (Buck and Burks, 1986). He and others found that this

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This dissertation uses the text style of the journal *Behavioral Neuroscience* as a model.

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compound specifically binds to a receptor on the C-afferent fibers and causes degranulation and degeneration of the fibers (Holzer, 1991). Nicolas Jancsó was intrigued by the myriad physiological consequences of chronic feeding of large amounts of Paprika to children. These physiological consequences have been reported to be associated with the neurotoxic activity of capsaicin. Among the effects of capsaicin are several that have reproductive implications due to the disruption of the C-fiber mediated sensory or neurohormonal stimulatory complex. In fact, capsaicin has become one of the most important of the tools that are used in the study of C-fiber innervation and the neuroendocrine complex associated with the nerves of the genitals. For example, capsaicin has been reported to inhibit implantation of the embryo into the endometrium, inhibit the analgesic effect of cervical stimulation, alter thermoregulation, and perhaps play a role in impotence in men (Traurig et al., 1984b; Traurig et al., 1988; Buck and Burks, 1987; Lazzeri et al., 1995).

The purpose of this study was to determine the potential effect of capsaicin on developing embryos. Capsaicin appears to have teratological and other deleterious developmental effects on newborn mammals. There is also evidence that it passes the blood/placenta barrier in rats, where it can affect the embryo (Scadding, 1980; Pellicer et al., 1996). C-afferent innervation is thought to play a role in the conduction of signals from the genitals and mammary nipples to the hypothalamus. The neural signals have been shown to trigger the release of various hormones. These hormones, in turn, have

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diverse endocrine functions related to pregnancy. We thought that capsaicin, under physiological conditions, might affect embryo development in several ways. First, capsaicin could bind to and influence the embryo itself before implantation, perhaps by delaying maturation and cell division. Second, we felt that disruption of the dams' C-afferent fiber innervation might affect the hormone balance enough to have a deleterious effect on the embryo. Although it is known that capsaicin may prevent implantation of the embryo, the effects of capsaicin on the post-implantation embryo were not as well studied. After implantation, the embryo is still maintained by a well-choreographed series of interacting hormones. We felt that capsaicin might alter these hormone pathways and cause teratological changes in the embryo.

The embryonics laboratory at Old Dominion University runs toxicology and quality control testing of instruments and media for human in vitro fertilization programs, using a mouse embryo co-culture assay (Ackerman et al., 1985). Thus, we have a unique capability for the assay of toxic compounds such as capsaicin for effects on embryo development. The mouse system has been proven to be an excellent model for effects on human embryos. Considering that the use of capsaicin is intensive and widespread, and that it has a specific and potent effect on nerves involved in neurohormonal processes of reproduction, we felt that there was a potential for defects or other alteration of embryo maturation, and that studies of the question posed were important.

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In order to gain a clear understanding of the ways in which embryo development might be affected by capsaicin, it is first useful to examine the processes of embryo development.

EMBRYO AND FETAL DEVELOPMENT IN THE MOUSE

Estrus, embryo development, and pregnancy in rats and mice are controlled by complex and interwoven hormone pathways. These pathways control the estrus cycle that prepares the reproductive structures for the initiation and maintenance of pregnancy, as well as the specific events that drive the development of the embryo from zygote to pup. The development of murine embryos itself is an indication of the state of the hormone balance of the reproductive system. The 21 days that the mouse embryo gestates in the uterus of its dam are the most precarious of its life. Any alteration or even fluctuation in the intricate balance of the endocrine balance of the dam (the female parent) can cause devastating deformities in the embryo. If there is any deviation in the complex and network of growth, implantation, and developmental factors, the embryo will not survive.

The hormones that control reproduction and pregnancy are derived from the gonads (testes, ovaries), the hypothalamus/pituitary stalk, as well as the embryo and other endocrine glands (Yen and Jaffe, 1991). In large part, the hormones that drive estrus and pregnancy are under hormonal feedback control.

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However, there is also a significant neuronal component to the complex that controls the endocrine system. The organs of generation, and of course the hypothalamus and pituitary, are richly innervated. This innervation is partially sensory, but even this part is capable of modulating the levels of certain of the key hormones of estrus and pregnancy (Sullivan et al., 1994; Peters et al., 1987; Besedovsky and Del Rey, 1996).

Genital innervation is quite complex and consists of many different types of neurons (Papka et al., 1987). However, an important component of the catalog of neurons types that serve the reproductive structures is the C-afferent fiber innervation (Traurig et al., 1984c; Papka et al., 1987). The C- afferent fibers have been implicated in a variety of important processes that control the endocrine system, and are an important component of the neurohormonal axis that controls estrus and pregnancy (Lazzeri et al., 1994; Nance et al., 1987; Papka et al., 1987; Traurig et al., 1988; Benson, 1994).

Oocyte development, estrus, and ovulation

Development of oocytes in mice begins in utero. The oogonia, which form from primitive ectoderm-derived germ cells that have migrated to the genital ridges, cease mitotic activity approximately 5 days before birth (Mandl, 1963). The developing oocytes initiate meiosis, but development is arrested in the diplotene stage of prophase I, which all the oogonia have entered by the fifth

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day post partum (Hogan et al., 1986). At birth, the oogonia begin to degenerate, so that at reproductive maturity, only approximately 10^4 oocytes remain, not all of which are mature (Hogan et al., 1986). The oocytes will not mature completely until the onset of the first estrus.

Estrus

Estrus behavior in the mouse corresponds with sexual maturity. The onset of estrus varies somewhat, the average being about six weeks of age (Green, 1966). At about four weeks of age, natural, cyclic rise in the titers of the sex steroids (estrone, estradiol) and the gonadotropins (lutenizing hormone (LH) and follicle stimulating hormone (FSH)) trigger hormonal changes that will result in sexual maturity and estrus. They also induce the changes in the reproductive organs associated with sexual maturity.

Estrus (Latin for "frenzy") is specifically the behavioral change that a female animal displays at the time of maximum fertility. This instinctive display is provoked by hormonal and physical changes that accompany ovulation. During estrus, a female will become receptive to a male and will attempt to mate.

Estrus in mice is periodic in nature, during which period the female reproductive organs are prepared for ovulation, mating and pregnancy. It corresponds in a general manner to the physiological changes that occur in the menstrual cycle in humans, although in humans the female is receptive

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throughout the cycle. In the absence of fertilization, the reproductive organs pass their optimal state and an anabolic process begins to dismantle the preparations for mating. The factors which control the periodicity of the estrous cycle are not entirely understood. It is known that the cycle varies considerably in length due to a variety of strain specific and environmental factors. Overcrowding, the presence of a male or male urine, and of course pregnancy can lengthen the period between estrous cycles. Stimulation with gonadotropins, which will be described in a later section, can truncate and reset the cycle. A second cycle then follows, often without completion of the first. There is evidence that the photoperiod at which the animal is maintained can affect the length of the estrous cycle, probably through the action of light on photoreceptor nerves connected to the medial preoptic region of the hypothalamus and its resulting effects on prolactin, progesterone, and estrogen (Lee et al., 1998; Yen and Pan, 1998; Rubin and Barfield, 1983; Houghton et al., 1997; Castro-Velazquez and Carreno, 1984). It should be noted that these hormones, particularly prolactin, are greatly affected by the action of the C-afferent fibers, and that activation of these fibers by stimulation of the cervix can initiate a condition known as pseudopregnancy (Kordon et al., 1994; Freeman and Banks, 1980; Whipple, 1989). Pseudopregnancy is the result of stimulation of the hypothalamic medial preoptic and dorsomedial and ventromedial areas of the hypothalamus as the result of C-afferent fiber stimulation of the cervix (Freeman and Banks, 1980). These regions control

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the nocturnal and diurnal prolactin surge, and by stimulation can be induced to stimulate release of large amounts of the hormone from the pituitary (Freeman and Banks, 1980). This surge in prolactin levels, along with associated increases in progesterone and other hormones, initiates a condition resembling pregnancy, including a decidual cell response (Kordon et al., 1994; Freeman and Banks, 1980; Whipple, 1989). Nonetheless, under optimal experimental conditions, a female mouse will enter estrus on the average of every four to five days. The course of the estrous cycle is thus controlled by a hormonal and neurological interaction between the hypothalamus and the ovaries. In response to elevated titers of gonadotropins released from the pituitary gland, maturation of the follicles is stimulated. These mature follicles release several hormones, notably estrogens, which inhibit maturation of other follicles by acting on the hypothalamus to regulate the release of gonadotropins. It has been observed that there is a correlation between stimulus of the C-afferent neurons in the cervix and uterus and release of gonadotropins from the pituitary (Kordon et al., 1994). These neurons are stimulated by introitus (Traurig et al., 1988; Sato et al., 1989). In fact, the female will prolong mating (lordosis) in order to maximally stimulate the sexual organs and ensure activation of the C fibers (Erskine, 1992; Erskine et al., 1989). C-afferent prolactin release may be stimulated, even in the absence of pregnancy or mating, by the stimulation of the mammary nipple (Traurig et al., 1984a; Flietstra and Voogt, 1996).

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Estrus may be divided into four different stages corresponding to events that occur at intervals during the cycle (Green, 1966, Hogan, Constantini, and Lacy, 1986). These stages are diestrus, proestrus, estrus, and metestrus. During diestrus, increasing gonadotropin levels, especially follicle stimulating hormone, stimulate growth and maturation of ovarian follicles. Certain of the more mature follicles, which are destined for ovulation, will begin to enlarge in response to an elevation of pituitary derived follicle stimulating hormone (FSH) levels. These enlarged follicles eventually differentiate from the immature follicles. FSH also promotes the maturation of the associated oocytes. In the uterus, the epithelium, which degenerated and was shed during the previous cycle, begins to re-grow and by the end of the stage is healthy and in good condition. In proestrus, the follicles that are destined for ovulation have reached their maximum size (about 550μm) and are clearly larger than those that are immature or destined to degenerate (average size 380μm). The enlarged follicles begin to secrete estrogens, which inhibits growth and development of immature follicles by indirectly inhibiting the release of FSH. Follicular mitosis stops. The hypothalamus stimulates the pituitary gland to release LH. In estrus, rising LH levels causes the maturing oocytes to break contact with their associated follicles and migrate to the periphery of the ovary. Lutenizing hormone stimulates final maturation of the follicle as well as increased estrogen production by the thecal cells in the mature follicle. The LH peak also initiates nuclear maturation of the oocytes. They undergo the first

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meiotic division and the first polar bodies are expelled from the oocytes. The epithelium of the uterus responds to the estrogens by beginning rapid cell division and by thickening in preparation for implantation and pregnancy. As LH concentrations reach their maximum, the eggs are released from the ovary into the proximity of the infundibulum. The uterine epithelium reaches its maximum thickness, and the distal portions of the oviducts enlarge to form ampullae. Cilia in the oviducts and muscular contractions of the uterus and fallopian tubes sweep the eggs into the ampullae. If fertilization is to be successful, mating takes place at this stage of estrus (Green, 1966; Hogan et al., 1986).

In the absence of mating, the estrous cycle enters its final stage, metestrus. The corpus luteum forms in the ovary and grows to maturity. The more mature non-ovulated follicles atrese. The eggs enter the oviduct proper, and leukocytes begin to infiltrate the epithelial lining of the uterus. Eventually, the epithelial lining is shed, the oocytes degenerate, and the cycle enters diestrus. Proestrus and estrus combined last about 48 hours. Metestrus and diestrus account for the balance of time during the cycle. The length of diestrus may vary considerably (Green, 1966).

Ovulation

The estrous cycle and ovulation respond to different hormonal signals. Apart from ovulation, the events in the estrous cycle are for the most part

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controlled by prolactin and the estrogens. Ovulation is directed by FSH and LH. Although estrus and ovulation are closely linked, they are not necessarily correlated. Under natural conditions estrus may take place without ovulation, as in young mice (Green, 1966). Ovulation may occur without estrus, especially when it is stimulated artificially with gonadotropins (Green, 1966). For the most part, however, ovulation takes place at the end of the estrus stage. During natural (non-stimulated) estrus, the ovaries in a female mouse may release eight to twelve eggs over a span of 1-3 hours (Hogan et al., 1986). Artificially induced ovulation under experimental conditions produces many more mature eggs and also induces the release of incompletely developed eggs. Under stimulation by human chorionic gonadotropin (hCG) and pregnant mare's serum gonadotropin (PMSG), release of eggs is more nearly simultaneous and thus the timing of the release is more precise.

As a follicle begins to grow in response to FSH, the oocyte begins a maturation process, which will only be completed after fertilization. As was mentioned above, the oogonia, which populate the genital ridges in the embryo, arrest development in prophase I of meiosis shortly after birth. In response to increasing size and maturity of the follicle, the oocyte now also increases in size from about 13μm to about 70μm. The follicle and the associated oocyte migrate to the center of the ovary to complete the development. As the levels of FSH fall and those of LH increase, the development of the oocyte and follicle enter a second phase. The follicle

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increases in size dramatically, with no associated increase in oocyte diameter. The oocyte resumes meiosis. The centrosome in the oocyte divides into two centrioles. The asters and the associated spindle structures are formed, and the oocyte proceeds through meiosis I to an uneven cytokinesis, resulting in the ejection of the first polar body. Meiosis II begins, but progress is stopped at metaphase II. The oocyte will normally remain in metaphase II until it is fertilized or it degenerates (Green, 1966; Hogan et al., 1986).

As the follicle attains its greatest size it begins to migrate to the periphery of the ovary. The follicle becomes a Graffian or antral follicle, with the antrum enlarged and filled with fluid. As the follicle reaches the periphery, the maturing oocyte erupts spontaneously in the vicinity of the infundibulum or open end of the ooducts. The eggs are swept into the ooducts and into the ampullae, where fertilization will take place (Green, 1966; Hogan et al, 1986).

During estrus, the female mouse becomes receptive to copulation with the male. Responding to instinctive drives triggered by the hormonal and neurological changes associated with estrus, she will actively seek to mate. The female mouse will in fact prolong lordosis for maximal stimulation of the cervix and vaginal areas (Erskine, 1992; Green, 1966). Coitus occurs at the beginning of estrus, with ovulation following 2-5 hours later. Mating usually takes place at about the midpoint of the dark cycle in animals that are maintained under conditions of diurnal light and darkness (Green, 1966; Hogan et al, 1986).

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Fertilization

During coitus in mice, some 10^7 sperm are released into the reproductive tract of the female. The male emits a coagulating substance from the coagulating and vesicular glands, which forms a vaginal plug and prevents the female from mating a second time (Green, 1966; Hogan et al., 1986). This vaginal plug traps some of the ejaculated sperm. Other factors prevent the vast majority of the sperm from entering the oviducts. Only a small proportion will arrive at the ampullae: perhaps as few as 10^2 sperm will survive and arrive to interact with the eggs (Green, 1966). Of the sperm that do reach the ampullae, some will arrive within as few as 15 minutes. Sperm motility appears to have little influence on this process, and it is more likely that the sperm are transported to the oviducts by the action of cilia lining the fallopian tubes and by peristaltic motions of the reproductive structures (Green, 1966). The sperm are not competent to fertilize the eggs until they undergo capacitation, a maturation process that requires the passage of approximately an hour to complete. By this time, most of the sperm that will interact with the oocytes have arrived in the ampullae and will come in contact with the oocytes as they enter the oviducts. Fertilization will take place in the ampullae or the distal portions of the oviducts. Sperm that do not interact with oocytes will remain within the reproductive structures and will be fertile for as long as six hours, and motile for as long as 13 hours (Green, 1966; Hogan et al., 1986).

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Sperm interact with and bind to receptors on the oocyte surface in a species specific manner. ZP3 receptors on the surface of the oocyte, in the cumulous mass and within the zona pellucida, bind with the sperm membrane (Bleil and Wassarman, 1980; Florman and Wassarman, 1985). This binding causes a reaction within the frontal portion of the sperm head that initiates the release of hydrolytic enzymes. This reaction, called the acrosome reaction, permits the sperm to digest openings within the proteoglycan matrix of the zona pellucida and penetrate towards the oolema, or cell membrane of the oocyte. In the absence of the acrosome reaction, the sperm are incapable of fertilizing the egg. The acrosome reaction is thought to be species selective, and is thought to involve a zonal protein known as ZP3 (Wassarman et al., 1996)

As a sperm penetrates the zona pellucida and reaches the oolema, it initiates a receptor mediated second messenger driven reaction within the egg called the cortical granule reaction. The cortical granule reaction involves the release of calcium into the oocyte matrix. This release of calcium, which can be simulated by treatment with an ionophore such as A-23187, initiates final maturation of the oocyte, facilitates the incorporation of the sperm genetic material, and prevents polyspermy. The cortical granule reaction causes the proteoglycans within the zona pellucida to cross-link, and induces changes in the zonal receptor proteins. These two events, called the zonal reaction, prohibit the penetration of further sperm by preventing binding of the sperm to receptors, by inhibiting the acrosome reaction, and by rendering the zona

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pellucida impervious to acrosome derived hydrolytic agents (Hogan et al., 1986).

Embryonic development to hatched blastocyst

During the cortical granule reaction, the head and midpiece of the sperm are incorporated into the oocyte. The sperm head disintegrates and the paternal genetic materials, centrioles, and mitochondria are added to what is now the zygote. Penetration of the sperm head also causes the oocyte to finish the second meiotic division and expel the second polar body. This ejection of the polar body takes place about three hours after fertilization. The polar bodies will remain in the perivitelline space until they disintegrate some time after the first cleavage. Temporary nuclear membranes form around both the male and female derived genetic material forming male and female pronucleii, where DNA replication takes place. These pronucleii will migrate to the center of the egg and will eventually form the basis for the metaphase plate. The pronucleii do not fuse; rather, the pronuclear membrane dissolve and the nuclear material condenses and lines up at the midpoint during metaphase of the first mitotic division (Green, 1966; Hogan et al., 1986).

The first cleavage of the embryo takes place about 24-30 hours after fertilization. Transcription takes place during this time, although much of the RNA and protein required for cell division is derived from the maternal

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complement supplied by the egg. The maternal RNA persists until the first cleavage, and then disappears rapidly (Hogan et al., 1986). Subsequent divisions transpire more frequently, with an interval of about 12 hours. The first two divisions (to four cell stage) take place as the embryo migrates through the oviduct. Subsequent cleavages occur in the uterus (Green, 1966).

Within five hours of the first cleavage, the embryonic cells enter the S phase of the second mitosis. The zygote divides symmetrically, and does not enlarge. Cleavage takes place within the zona pellucida, which prevents adhesion of the embryo.

About the third day post-coitus the embryo has divided into a compacted sixteen or more cell mass called a morula. At this time the embryo begins to enlarge and a fluid filled cavity forms within the ball of cells called a blastocoel. On day four, the blastocoel has fully formed and the embryo has reached the blastocyst stage. The mature blastocyst contains approximately 65 cells (Hogan et al., 1986). By this time the embryos are randomly dispersed within the horns of the uterus due to peristaltic motions of the uterine muscles. At approximately $4^1/_2$ days, the zona pellucida opens and the blastocyst hatches. The cell mass of the blastocyst is extremely adhesive and sticks to the uterine wall (Green, 1966).

Until the morula stage, all the cells in the mouse embryo appear to be equipotent. At the sixteen cell stage, however, there is a division in the potency of the cells. By the time the embryo develops into a blastocyst, a

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trophectoderm cell lineage and an inner cell mass (ICM) have differentiated (Hogan et al., 1986). The trophectoderm will give rise to the extraembryonic tissues such as the placenta and the allantois, while the ICM will develop into the embryo. The reason for this polarization of developmental potential appears to be due to changes in the genome that take place in morula stage cells (Hogan et al., 1986).

Implantation

The hatched blastocyst adheres to the epithelium of the uterus and induces the formation of a crypt into which the embryo settles. This process is assisted by preparation of the uterine epithelium by ovarian hormones, especially progesterone (Hogan et al., 1986). In the absence of those hormones, the uterine epithelium will not favor implantation. In fact, the uterus and blastocyst must reach the proper stage of maturation at the same time (circa day 5) for the embryo to bind and implant successfully (Green, 1966). The trophoblast cells (from the trophectoderm) invade the uterine epithelium and enter the endometrium (Hogan et al, 1986).

The implantation of the embryo into the endometrium initiates an inflammatory event called the decidual response. This response is progesterone and prolactin dependent, but otherwise rather non-selective, as the decidual response may be triggered by adhesion of any irritant substance to the uterine

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epithelium (Hogan et al., 1986). It should be noted that there is evidence of a neurohormonal complex which controls the decidual response and probably involves C-afferent fiber (Traurig et al., 1988). The decidual response is an inflammatory event, and C-afferent fibers have been shown to promote inflammation (Ratzlaff et al., 1992). The initiation of the prolactin surge by cervical C-fiber stimulation has been linked with the endometrial changes that are necessary for the formation of the decidual complex (Traurig et al, 1988). Among the events associated with the decidual response is an increase in the mitotic activity of the endometrial cells. This causes a thickening of the endometrium and a complete enveloping of the embryo and trophoblast, eventually forming a layer that separates the embryo from the uterine lumen (Green, 1966). The blood vessels in the basal sections of the endometrium reform into blood sinuses, which invade the inflammatory site and supply it with nutrients (Green, 1966). Part of this increased blood flow is evoked by substance P secreted from the C-fiber innervation (Gram and Ottesen, 1982). The decidual tissues eventually differentiate into distinctive cell types, contributing to the formation of the placenta and the membranes surrounding the amnion (Green, 1966).

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Post-implantation embryo development

The implanted trophectoderm differentiates into several structures in the mouse embryo. Early in the blastocyst stage the trophoblast forms a primitive epithelial tissue (Hogan et al., 1986). After implantation, this epithelium evolves into specialized tissue, depending on the region of the embryo with which it is associated. The position of the inner cell mass in relation to the trophectoderm is especially important in determining the eventual fate of the tissue type (Hogan et al., 1986). Parts of the trophectoderm will eventually become the extraembryonic ectoderm, the chorion, and the major portion of the placenta (Green, 1966; Hogan et al., 1986).

The ICM of the blastocyst begins to differentiate after implantation. By the sixth day post-coitum, the ICM forms a central cavity and begins to form a primitive epithelium from the ectoderm cells which surround it. The embryo, which is still in blastocyst stage, begins gastrulation and becomes a gastrula. By late the seventh day neurulation has begun, the notochord and primitive streak have formed. The primary trophoblast giant cells have given way to the parietal endoderm, Reichert's membrane is present, and the amniotic fold begins to appear. On day 8 the amnion and amniotic cavity are evident, as is the head process and blood islands. The ectoplacental cavity forms, as does the allantois. Somites have appeared and number about seven. By day nine the heart and the fore gut pocket are present. The blood islands turn into

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portions of the peripheral circulatory system. The chorion forms. The neural tube is well developed. The mouse embryo folds over upon itself and assumes the C shape it will retain for the balance of gestation (Green, 1966). By day eleven, the mouse embryo has developed the beginnings of many of the major organs, and is now considered a embryo. It is important to note that most of the structures that will give rise to the nervous system appear on days 8, 9, and 10. These three days are important for testing of teratogenic and neurotoxic agents, as limb-bud formation occurs at this time and the embryo is particularly sensitive (Green, 1966; Hogan et al., 1986).

Important aspects of embryo development regarding the role of innervation

There is ample evidence of a connection between the nervous system and the hormone releasing tissues that control pregnancy. As was mentioned earlier, nerves connected to the cervix and uterus have been shown to stimulate release of prolactin from the pituitary (Freeman and Banks, 1980; Kordon et al., 1994). Afferent C fibers of the sensory nervous system have been implicated in many of the neurogenic effects associated with reproduction.

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NEURONS AND THEIR ROLE IN REPRODUCTION

C-afferent fibers and their importance

C-afferent nerve fibers are small (about 0.2-1.5 (m in diameter), monopolar primary afferent neurons (Buck and Burks, 1986; Holzer, 1992). They are the smallest of the neuron types and are unmyelinated. Their conduction rate is comparatively slow. Afferent C fibers are polymodal nociceptors, and conduct sensation of warmth and mechanical stimulation (pressure, vibration). They are also important in thermoregulation (Pellicer et al., 1996). They are reported to contain the neurotransmitter substance P, which is an identifying marker (Holzer, 1992; Fitzgerald, 1983). This substance P is an undecapeptide also found in the central nervous system (Kanazawa and Jessel, 1976). Substance P is known to play a role in neurogenic inflammation and is suspected of having a role in immunogenic inflammation (Ratzlaff et al., 1992). SP has also been implicated in a number of other inflammatory processes such as arthritis.

C-afferent fibers are found in all epidermal tissues, and are notably prevalent in sex organs (Pinter and Szolcsanyi, 1995; Nilsson and Brodin. 1977; Skrabanek, and Powell, 1983; Papka et al., 1985; Holzer et al., 1982). There is extensive C-afferent fiber innervation of the cervix, uterus, vagina, bladder, and ureter in the female mouse (Traurig et al., 1984c). The pelvic region is innervated by the pudendal and pelvic nerves. The pelvic and

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pudendal nerves supply the C-fibre innervation, although not, apparently, all the reproduction related innervation (Komisaruk et al., 1997).

C-afferent fiber innervation can be blocked by the action of capsaicin. Capsaicin, a member of a small, pungent family of botanical compounds knows as vanillamides, has been shown to bind specifically to receptors on C afferent fibers, and to cause degranulation and release of SP (Theriault et al., 1979; Gamse et al., 1981).

CAPSAICIN, C-AFFERENT FIBERS, AND REPRODUCTION

History of capsaicin and Capsicum

Capsaicin (8-methyl-n-vanillyl-6-nonenamide, Figure 1) is the most potent and most concentrated of the several pungent agents found in red or hot peppers (Govindarajan and Sathyanarayana, 1991).

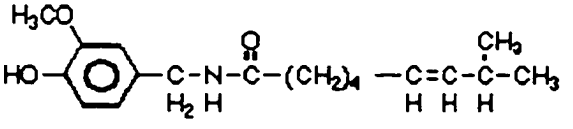

Figure 1. Capsaicin. (8-methyl-n-vanillyl-6-nonenamide).

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Capsicum peppers (*Capsicum annuum* and *Capsicum frutescens* and related species, Family *Solanaceae*) are native to Central and South America, where they have been used in foods and in medicinal preparations since prehistoric times. The Aztec peoples of Central America and Mexico considered capsicum a ritual food and used it in their religious practices (Dasgupta, and Fowler, 1997; Diaz del Castillo, 1963; Lembeck, 1987; de La Vega, 1966). The Inca peoples of Peru even worshipped the red pepper as one of their creator gods, Ayar Uchu (de la Vega, 1966). Many of the native foods of Central and South America, for example mole, contain capsicum fruit as a spice (Dasgupta and Fowler, 1997).

Capsicum was brought to Europe by Columbus during his second voyage in 1493 (Lembeck, 1987). From Spain, the use of hot peppers spread throughout Europe, mainly as a substitute for the hard to obtain black pepper, and was employed in the preservation of meat as well as in medicines. From Europe the use of capsicum spread throughout the Spanish empire to the far east, and today, hot peppers are an ingredient of foods and medicines throughout the world (Dasgupta and Fowler, 1997).

Environmental exposure to capsaicin

Capsaicin is most commonly encountered in three types of preparations: foods, medicines, and defensive sprays. Other sources of exposure are

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agricultural (harvesting of capsicum fruit) and industrial (spice preparation and extraction of capsaicin for medicinal and other use). These preparations use more or less concentrated extracts of capsicum fruits, and can be the source of chronic and acute exposure in humans.

Food preparations

Capsicum fruit is popular throughout the world as a food and a condiment. The levels of capsaicin found in these fruits ranges from none in bell peppers to about 1-2% capsaicin in habanero peppers (Govindarajan, and Sathyanarayana, 1991; Dasgupta and Fowler, 1997). Certain peppers, the black mombasa pepper from Africa, for example, have capsaicin levels so high that they cannot be eaten undiluted due to their vesicant nature; they blister the mouth tissues (Lembeck, 1987).

The capsicum or chili (or chilli, or chile, or red pepper; also Agi or Aji) has been used by native Americans in the regions of Central and South America from at least 5000 BC. The general use of hot pepper in Spanish cooking today is illustrated by the refrain "Ajo, sal, y pimiento, y lo demás es cuento" (garlic, salt, and hot pepper; everything else is trivial). Capsicum plays an important role in Central European cooking (the Hungarian "Paprika"), in Indian and Eastern foods (Korean Kimchi, for example), and in the hot, spicy foods of the Southern and Southwestern United States (Lembeck, 1987; Govindarajan, and

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Sathyanarayana, 1991; Dasgupta and Fowler, 1997). In Russia, capsicum peppers are added to vodka to make a fiery concoction called Pepperovka.

There are, in addition to foods in which the fruit of the capsicum plays an important role, a host of sauces and other condiment preparations which use extracts of hot peppers. These vary in potency and often are advertised emphasizing their potency. The capsaicin content of some food preparations can be quite high. Although Capsicum frutescens fruit can contain as much as 2% capsaicin, sauces containing oil or vinegar extracts of these peppers may be much higher in capsaicin content due to concentrating effects of the preparation process.

Medicinal preparations of capsaicin

The use of capsicum peppers as medicine is prehistoric. The Incas of Peru used it for its medicinal properties (de la Vega, 1966). The US Pharmacopoeia has listed capsicum as a medicine since at least the middle of the last century (Wood and Bache, 1854). Home-made, over the counter, and prescription drugs containing capsaicin are widely used throughout the world for a variety of ills. Capsid and Zostrix are two prescription medications containing capsaicin in an emmolient base for topical use as an anti-inflammatory and pain medicine for the treatment of arthritis. Capsaicin is readily absorbed through the skin, especially when dissolved in an oil solution or in DMSO. Capsicum

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preparations were and probably still are used internally Per Os (by mouth) as "vermifuge, carminative, counterirritant, and stomachic" (Wood and Bache, 1854). Capsaicin has even been used to cure impotence, by injection into the urethra. This latter use is certainly a testament to great fortitude (Lazzeri et al., 1994)!

Defensive sprays

With the advent of widespread gun control, defensive pepper gas or oil sprays have become popular. Police and civilians use canisters of spray containing capsaicin extracts for defensive uses, as well as for crowd and prisoner control. These defensive pepper gas preparations have become the most prevalent form of non-lethal defense. Pepper gas units have a typical capacity of one to five ounces (30-150 grams) and with a capsaicin concentration of 3-15%. The solvent used in these sprays varies, but may contain ethanol, a water based detergent solution, or some other organic solvent such as dimethyl sulfoxide (DMSO). The capsaicin is readily absorbed through the skin, especially if dissolved in organic solvents.

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Industrial exposure to capsaicin

Contact with the fresh fruit of the capsicum can cause irritation of the skin (Jancso et al., 1967,). Repeated contact, as occurs when capsicum fruit is harvested, can increase capsaicin exposure in farm workers. Capsaicin containing residue of hot peppers can be highly irritating, especially in mucous membranes and in the eyes.

Pharmacology of capsaicin

Capsaicin attenuates nerve sensation in the mouth and digestive tract. Upon repeated exposure increasing dosages may be eaten as a person becomes desensitized and builds tolerance. Capsaicin is readily absorbed by the digestive tract, and, although the greater part is destroyed by the liver, as much as 5% of the capsaicin enters into the blood system (Donnerer et al., 1990). Humans can be exposed to quite high dosages of capsaicin either through ingestion or skin contact. Capsaicin is neurotoxic at dosages less than 50 mg/kg in laboratory animals. A 100 kg human who eats one ounce (32 grams) of the hottest edible peppers will exceed this dosage (>500 mg). It is lipophilic, and passes readily through the lumen of the intestine (Monsereenusorn, 1980, Donnerer et al., 1990; Kawada et al., 1984). Enough capsaicin can enter the blood that chronic intake can have noticeable

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physical and psychological effects (Whipple et al., 1989; Cheng et al., 1995; Cormareche-Leydier, and Vernet-Maury, 1989; Alleva et al., 1991; Szikszay et al., 1983; Nance et al., 1987). Once the capsaicin enters the bloodstream, it readily interacts with all portions of the body, crossing both the blood/brain barrier and the blood/placenta (Saria et al., 1981; Saria et al., 1982).

Mechanism of capsaicin neurotoxicity

Capsaicin interacts with VR1 receptors located on C-afferent sensory nerve fibers, causing activation and subsequent loss of nerve function (Meini et al., 1992; Caterina et al., 1997; Clapham, 1997). The capsaicin receptors on C-afferent fibers were described by Meini et al. as the receptor for the endogenous agonist lipoxin A4 (Meini et al., 1992). C-afferent fibers are warmth and pressure receptors and polymodal nociceptors, and have been implicated in a variety of roles in mammals. Capsaicin activation of C-afferent fibers causes an initial stimulatory reaction with associated hot pain sensations. At higher dosages, capsaicin induces a massive release of neuropeptides such as substance P from the fibers, resulting in depletion of these neurotransmitters (Nagy et al., 1980). Capsaicin also appears to inhibit recovery of the neuron function by blocking axonal transport within C-afferent fibers (Kessler and Black, 1981; Otten et al., 1980). The degranulation of the C-afferent nerve fibers causes a loss of sensory information from those fibers due to failure of the

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nerve function. The inhibition of nerve function caused by capsaicin in adult mice can last up to six weeks (Fitzgerald, 1983). Newborn rats and mice treated with capsaicin permanently lose C-afferent nerve function (Holzer, 1991; Cuello et al., 1981). This loss of C-afferent function persists into adulthood, is irreversible, may affect animal viability, and certainly affects fertility (Pellicer et al., 1996). The loss of C-afferent function in rats treated with capsaicin at birth is linked to degeneration of the nerve fibers. The mechanism of this degeneration is unknown, but is perhaps related to loss of axonal transport (Holzer, 1991; Kessler and Black, 1981; Otten et al., 1980).

Effects of capsaicin neurotoxicity

Capsaicin induced loss of C-afferent nerve function is expressed in two ways. First, the nerves are unable to transmit sensory information. The perception of heat and mechanical stimulation is lost, resulting in numbness. C-afferent fibers appear to be involved in initiation of neurohormonal copulatory reflex in both male and female rats (Traurig et al., 1988). Rats treated with capsaicin as neonates have reduced fertility as adults (Traurig et al., 1984b; Traurig et al., 1988). The neurohormonal reflex, which is initiated by copulation, is carried by C-afferent fibers, and is reduced in the capsaicin treated rats. There is a reduced secretion of luteotropic hormones in capsaicin treated rats, implantation of embryos is impaired, and there is inadequate

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hormonal support for pregnancy. Negative effects of capsaicin pretreatment on fecundity have been described (Pellicer et al., 1996). C-afferent fibers also have a role in signaling tumescence in human males (Lazzeri et al., 1995). The second mechanism by which capsaicin induced loss of C-afferent function is expressed in the massive release and subsequent depletion of neuromodulators such as substance P (Gamse et al., 1980). Substance P is the active agent involved in neurogenic inflammation; it directly induces plasma extravasation in perineural blood vessels, and triggers mast cell degranulation, which results in an inflammatory "triple response" of weal, flare, and erythema (Jancso et al., 1967, Holzer, 1991). Substance P released by the C-afferent fibers is thought to enhance the inflammation triggered by IgE mediated inflammation, and may also be the active irritant in nervous inflammatory conditions, such as nervous hives (Holzer, 1991). When injected into an adult mouse, capsaicin activates C-afferent fibers and triggers massive release of neuropeptides (Jancso et al., 1967). The animals must be injected under general anesthesia or they succumb to systemic shock. The loss of C-afferent function can persist up to two weeks, although the immediate inflammation and burning sensation lasts only about ten minutes (Jancso et al., 1967).

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SIGNIFICANCE AND SPECIFIC AIMS OF THE RESEARCH

The effects of capsaicin upon C-afferent fibers appear to be a general mammalian reaction. Perinatal injection of capsaicin in humans has not been studied. However, the effect of low dose injection in the skin of adult humans mimics that of similar injection in rat (Jancso et al., 1967). The extrapolation of the effects of perinatal capsaicin injection from rats to humans seems reasonable; the effects of this agent on fetal and suckling mice could give an indication of its effects on children. Although the effects of treatment of neonate rats and mice with capsaicin have been extensively described, the effect of in utero exposure to capsaicin of rats or mice has only recently been reported (Pellicer et al., 1996). It has been demonstrated that rat embryos have active capsaicin receptors and functional C-afferent fibers after the second week of gestation (days 13-17). Nervous system development begins on days 8 or 9, and thus the potential exists for capsaicin effects on embryo development in the pre or early post implantation embryo. The effect of capsaicin on early or late stage embryo development was unknown. Further, we know that rats treated with capsaicin as newborns have reduced fertility as adults due to problems with implantation (Traurig et al., 1988). However, it is not known whether adult rats treated with capsaicin also evince reduced fertility. Is the neurohormonal pathway altered by capsaicin treatment to such an extent that development is

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compromised? That is what the assays described herein were designed to answer.

Research aims.

Capsaicin has been shown to inhibit the development and cause degeneration of nerve fibers when injected into neonate mice (Nagy et al., 1981). The concentrations of capsaicin that can be encountered in the environment are quite high; in certain preparations, it can exceed by ten-fold the dosage which has been found to be toxic in laboratory animals. The effects of perinatal capsaicin on fertility, embryo development, fecundity, and generative organ morphology have been only superficially studied. Considering the fact that humans, including pregnant women, are chronically and acutely exposed to capsaicin, we felt a close look at the effects of capsaicin on the above was justified.

Specific Aim: Determination of the effect of perinatal capsaicin exposure on embryo development and fertility.

The specific aim was divided into three sub-aims. Two of these examined a stage, from early to late, in embryo development. The third was done for background purposes. Capsaicin uptake by mice via several routes was

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measured. This capsaicin uptake assay was performed in order to determine the potential for toxic reactions from exposure to capsaicin.

Embryo co-culture. The fertilized eggs were exposed to capsaicin and examined for effects of this exposure on early embryo development. The zygote was followed as it matured from two-cell through four-, and eight-cell stages, and then to morula and blastocyst stages.

Teratology by fetus clearing. Pregnant mice were treated with capsaicin in clinical dosages. The pregnancies were allowed to progress to near term, at which time the dams were sacrificed and the fetal pups examined for any teratological changes in the development of bone and cartilage.

Uptake of capsaicin by mice. There are several possible routes of capsaicin exposure: ingestion, topical application, and injected. Mice were exposed to capsaicin by these three routes, and blood analyses were performed to determine serum capsaicin concentrations.

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MATERIALS AND METHODS

It is well known that capsaicin causes destruction of C-afferent fibers in neonate rats and mice (Holzer, 1991). However, there was no data concerning the possible effects of capsaicin on embryo development, and whether C-afferent fibers are destroyed by treatment with capsaicin in utero. Further, although it has been established that neonatal capsaicin treatment has detrimental effects on implantation and pregnancy support in rats, there was no data concerning effects on fertility following acute adult capsaicin treatment in rats. Although it is unlikely that anyone would expose newborn infants to high doses of capsaicin in any form, pregnant women can be exposed in several ways, thus also affecting their embryos. Thus, we looked at the different routes of potential capsaicin absorption, and measured the amounts of capsaicin actually absorbed. Then, we studied the effects of capsaicin on embryo development at several stages in a pregnancy. We looked for detrimental effects of capsaicin on embryonic development by co-culture of capsaicin with embryos from two-cell to blastocyst stages. Finally, pregnant female mice were treated with capsaicin, and the fetuses examined for teratogenic effects by fetal clearing.

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ANIMALS

Strains

Two strains of mice were used in this project. CD-1 mice, originally derived from Charles River Laboratories (Wilmington, MA), were used for determination of the capsaicin levels in the blood and also for the fetal clearing assays. CD-1 is an inbred strain of white/albino mice and is commonly used in laboratory procedures. The mice used in the experiments were bred in-house in the colony in the animal facility at Old Dominion University. All the CD-1 female mice used were from eight to twelve weeks old and ranged in weight from 20–40 grams. CD-1 mice were used as proven studs for breeding. These were up to six months old.

For the embryo coculture assays, the strain B6CBAF1/J was used. This is a crossbred strain of C57BL/6 females and CBA males. The B6CBAF1/J mice were obtained at eight weeks of age from Jackson Laboratories (Bar Harbor, ME), and acclimated to the Old Dominion University animal facility for one week prior to use. B6CBAF1/J mice were selected for the embryo coculture assays for several reasons. They are currently being used by this laboratory for media and instrument coculture toxicity studies related to in vitro fertilization, and there is a great deal of data on this strain. Second, they are known to ovulate consistently and productively in response to hormone stimulation. Third, their embryos have

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been shown to react to toxic agents as do CD-1 embryos. CD-1 embryos, however, do not culture as consistently in vitro as do the embryos from B6CBAF1/J mice. Mating of the B6CBAF1/J for embryo collection was done using proven stud CD-1 males (Ackerman et al., 1985; Ackerman et al., 1983).

Rationale for the use of animals

The use of animals is essential to the determination of a system as complex as the one being studied. Neither embryo coculture nor the development of mice can be modeled by the use of neither tissue culture, computer modeling, nor by any other currently known alternative method. The selection of the CD-1 and B6CBAF1/J/J mice is in order to continue established procedures (Ackerman et al., 1985; Ackerman et al., 1983). The numbers used were required for statistical purposes.

Care of the animals

The animals were maintained in an accredited university animal facility staffed by fully trained personnel and were under veterinary care. The animals were environmentally protected, disease free, fed laboratory rodent chow (Ralston Purina, St. Louis, MO.) and watered *ad libitum*. All the mice were maintained in a diurnal 14 hour light: 10 hour dark regime, with the dark cycle beginning at 17:00

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hours. The temperature was maintained between 21C and 27C, except for a short span of time when equipment failures caused an increase in the room temperature to 30C for several days.

Anesthesia

All the animals used in this experiment were anesthetized at the beginning of any procedure requiring survival, and remained under anesthesia usually until the animal was euthanized. Any pain was limited to intraperitoneal injections of anesthetic. In most cases the anesthetic used was pentobarbital (64.8mg/kg, Anpro Pharmaceutical, Arcadia, CA). However ether inhalation (Fisher Scientific, Pittsburgh, Pennsylvania) was used upon occasion for procedures of short duration and for euthanasia.

Euthanasia

Euthanasia of mice was performed by cervical dislocation, inhalation of carbon dioxide, or ether overdose. All euthanasia and animal use and care procedures are fully in accordance with established procedures and were consistent with guidelines found in *NIH Guide for the Care and Use of Laboratory Animals* and approved by the panel on Euthanasia of the American Veterinary Medical

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Association. All animal procedures were also approved by the Old Dominion University Animal Use and Care Committee before the experiments were performed.

EFFECT OF CAPSAICIN ON PRE-IMPLANTATION EMBRYO DEVELOPMENT

Capsaicin effects on pre-implantation embryo development were studied by embryo co-culture. This method was pioneered by Ackerman and Swanson for use as a quality control tool for screening instruments and media used in human In Vitro fertilization clinics (Ackerman, and Swanson, 1983; Ackerman et al., 1984; Ackerman et al., 1985; Swanson, and Leavitt, 1992). Female mice were superovulated and mated. After fertilization, the mice were sacrificed and the embryos collected. The embryos were placed in culture media in presence and absence of capsaicin. Development of the embryo was followed as it divided from two cell to blastocyst stages.

Superovulation

Female B6CBAF1/J mice were injected intraperitoneally (IP) with 5 international units (IU) of pregnant mare's serum gonadotropin (PMSG, Sigma) to stimulate overproduction of ovarian follicles (follicular recruitment). Forty-eight hours later, the mice were injected with 5 IU human chorionic gonadotropin (hCG, Sigma) to stimulate follicle maturation and ovulation. The

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females were placed in a cage with a proven fertile CD-1 male. Sixteen hours after hCG injection, the females were separated from the males, and examined for vaginal plugs, which indicated successful mating (plug positive).

Extraction of embryos

The plug positive female mice were sacrificed 24-28 hours later and the oviducts were extracted and placed in Krebs (made in-lab, see Appendix A) medium with 2mg/ml bovine serum albumin, fraction V added (Krebs/BSA, BSA from Sigma), 2ml in a 35mm sterile petri dish as described in the literature (Ackerman and Swanson, 1983; Ackerman et al., 1983; Swanson and Leavitt, 1992). The plates were placed under a dissecting microscope (Zeiss-Urban Quadrascope, Zeiss Co., West Germany) and a 30 gauge needle (Becton Dickinson & Co., Rutherford, NJ) attached to a 1ml syringe (Becton Dickinson) filled with Krebs/BSA was inserted into the fimbriated end of the oviducts. The embryos were flushed into petri dishes containing Krebs/BSA, and normal two cell embryos were collected.

Coculture

The collected embryos were inspected under the microscope for defects, and the healthy ones were allocated to wells in Costar 96 well cell cluster plates

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(Costar Corporation, Cambridge, MA). The wells contained the following treatments. Control embryos were placed in Krebs/BSA. A second control group was placed in Krebs/BSA with 1% ethanol (95% medical grade, AAPER Alcohol and Chemical Company). A 300 mM stock solution was made of capsaicin in the 95% ethanol, and was diluted in fresh alcohol to make the different concentrations, so that the concentration of ethanol remained constant. As a preliminary study, serial dilutions from 3mM to 0.3μM were made of capsaicin in Krebs/BSA with 1% ethanol, and the embryos were assayed in these media. In order to more closely determine the concentration of capsaicin at which the embryos were affected, embryos were placed into solutions of Krebs/BSA with 1 % ethanol and 3mM, 2.2mM, 2mM, 1.5mM, 1.3mM, 1.1mM, and 1mM capsaicin.

The embryos were cultured at 37C in 5% CO_2 in air, 100% humidity, and examined for developmental defects every 24 hour period for the next five days (Swanson and Leavitt, 1992).

Determination of embryo development and statistical analysis

On day five PC the plates containing the embryos were removed from the incubator and the embryos examined under an inverted microscope (Nikon Corporation, Japan) for developmental progress. The embryos were then scored according to their progress on that day. The score was as follows. A

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score of 1 was given to 2-3 cell embryos; a score of 2 was given to four to six cell embryos; a score of 3 was given to 7-9 cell embryos; a score of 4 was given to 10 cell- morula and other pre-blastocyst embryos, and a score of 5 was given to blastocysts, whether they had hatched or not. Embryos that degenerated before day 5 were given a score of 0. The scores recieved by the embryos were grouped by treatment and averaged. The average scores for each treatment group were analyzed using the Chocran-Mantel-Haenszel (CMH) test, which is specific for ranked as opposed to measured data. The calculation of the CMH test was performed on a mainframe version of SAS.

EFFECT OF CAPSAICIN ON POST-IMPLANTATION FETAL DEVELOPMENT

Pregnant female CD-1 mice were subjected to acute capsaicin exposure at several stages during pregnancy, and their fetuses were examined for abnormal development. Effects of capsaicin on fetal development was examined in two ways. Effects of capsaicin on bone and cartilage development were examined by fetal clearing and staining of the bone and cartilage.

Mating, natural estrous cycle

Female CD-1 mice were presented to proven stud male CD-1 mice in their natural estrous cycle (non-hormonally stimulated). Sixteen hours after

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presentation the females are examined for vaginal plugs, which verified mating. Plug positive females were set aside and labeled for date of insemination. Females that failed to mate were kept with the stud male for up to five days (one full estrous cycle).

Treatment groups

Capsaicin was dissolved in ethanol (95% medical grade, AAPER Alcohol and Chemical Company) to make a 300mM (10%) stock solution. The stock solution was used to make dilutions with a vehicle for injection. The vehicle used was 80% phosphate buffered saline (pH 7.4) with 10% TWEEN-80 (Sigma) and enough ethanol to give a final ethanol concentration of 10% (EPT). Vehicle alone was used as a negative control. EPT containing 55% retinoic acid was used as a positive control, as this substance has been reported to have teratogenic activity (Kochhar, 1973; Kochhar et al., 1996). Plug-positive females were randomly placed into one of six groups, according to treatment. The females were injected SQ under the dorsal skin on three concurrent days, from days 8-10, with 100 (l EPT containing the following solutes: (Table 1).

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Table 1
Treatment groups for fetal clearing assay.

Group: Treatment	Injection Solution
Group 1: negative control (-CON)	Injected with EPT
Group 2: positive control (+CON)	Injected with EPT/ 0.55% retinoic acid
Group 3: 3.3 (moles capsaicin	Injected with EPT/ 33mM capsaicin
Group 4: 1.6 (moles capsaicin	Injected with EPT/ 16mM capsaicin
Group 5: 0.6 (moles capsaicin	Injected with EPT/ 6mM capsaicin
Group 6: 0.3 (moles capsaicin	Injected with EPT / 3mM capsaicin

All injections in 100µl EPT

Injection schedule and rationale

Days 8, 9, and 10 post coitum are critical to limb bud and other structural development, and so teratogenic agents are especially effective at this time (Kochhar et al., 1996). At day 17, the pregnant female mice were injected with an overdose of pentobarbital, and the fetuses were removed. The fetuses were then stained for bone and cartilage examination as described by Wassersug (Wassersug, 1976). The pups from each female were kept in separate urinalysis containers (unknown provenance) marked with the dam's number, the date of treatment, and the type of treatment.

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Fixation, clearing, and staining

The pups to be stained were placed in 10% buffered formalin (pH 7), and fixed for 48 hours. The pups were then eviscerated and partially flayed. They were washed in five changes of distilled water, and placed in a cartilage staining solution of 10 mg Alcian blue 8GX (Lipshaw Manufacturing, Detroit, MI) in 70ml absolute ethanol and 30 ml glacial acetic acid (Fisher) for 8-12 hours. The pups were washed in absolute ethanol, and dehydrated by immersion in three washes of absolute ethanol for 24 hours each. The pups were re-hydrated in decreasing concentrations of ethanol (75%, 50%, and 25% ethanol, 24 hours each), and washed twice in distilled water, 24 hours each wash. They are macerated in enzyme solution (1% trypsin (Fisher) in 70 ml distilled water with 30 ml saturated sodium borate (Fisher) solution) for 1-2 hours until the soft tissue was jelled. This processes was accelerated by maintaining the solutions on a hot plate at 40 C. The times for completion of the maceration process varied widely. The pups were washed in distilled water and placed in a bone staining solution (0.5 M potassium hydroxide, Fisher, with 0.1% alizarin red (Sigma) added dropwise until the color turns deep purple) for 48 hours. The pups were cleared by immersion in 25% glycerol (Fisher) containing 0.5% KOH for several days, followed by a wash in 50% glycerol/0.5% KOH for 24 hours, a wash in 75% glycerol/ 0.5% KOH for another 24 hours, and finally 100% glycerol for 24 hours. The pups were stored in

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fresh 100% glycerol, and examined for bone or cartilage defects under a dissecting microscope (Wild Corporation, Heerbrug, Switzerland). The fetuses were measured from crown to rump (see Figure 2), and their weight was obtained with an analytical balance. After clearing, bone and cartilage staining, the fetuses were examined under the microscope to determine bone or cartilage deformities. The ribs, head, pelvis, and tail, as well as the right paw and the left foot were examined for deformities and the bones (carpals and falanges) of the hand and foot were counted. The length of the humerus and ossification of the same were measured. Comparisons of weight or length between treatment groups was done using the following formula:

$$S_{experimental} / \text{Average } S_{control} = S_{corrected}$$

Where S is the length or weight of the fetuses. $S_{corrected}$ was used for all calculations involving weight or length. Statistical comparisons were performed using the analysis of variance (ANOVA) program in Instat (Graphpad).

BLOOD LEVELS OF CAPSAICIN AFTER EXPOSURE BY DIFFERENT ROUTES

Female mice were treated with capsaicin by the three possible routes of exposure: by feeding capsaicin in food; by exposing the skin to capsaicin in solution; and by injection. The mice were then sacrificed and bled. The blood tissues were

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assayed for capsaicin and capsaicin levels were measured to determine the potential of capsaicin toxicity.

Routes of exposure of animals to capsaicin

Oral

Capsaicin (Sigma Chemical Company, St. Louis, MO) was dissolved in absolute ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY) to make a 300mM stock solution, which was diluted in appropriate solvents to levels used for exposure. Mice were fed capsaicin by the use of a gavage tube. The mice were anesthetized with pentobarbital, and a dose of capsaicin of 3μmoles (approximately 50-100mg/kg), as a 30mM solution of capsaicin in olive oil (with 10% ethanol) was injected directly into their stomachs. Alternatively, the mice were tranquilized with acepromazine or pentobarbital and the mice were fed the capsaicin/oil solution by mouth using a syringe to deposit the oil on their tongues and allowing them to swallow it.

Topical application

Capsaicin stock solution was dissolved 1:10 in dimethyl sulfoxide (DMSO, Fisher) to make a 30mM capsaicin: 89% DMSO: 10% ethanol solution

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for topical exposure. The treatment volume was 100 µl, with a total dosage of 3µmoles. The mice were anesthetized with pentobarbital, and the abdomen was shaved. The Capsaicin/DMSO solution was applied with an Eppendorf pipette (Eppendorf Company, Germany) and distributed with the pipette tip.

Injection

For sub-cutaneous (SQ) injection, a 30mM solution of capsaicin in EPT was used. The solution was injected into the mice, at a volume of 100 (l/mouse, to give a 3µmole dosage. 3µmole/mouse (about 50-100mg/kg) capsaicin has been shown to cause depletion of Substance P from C-afferent fibers in laboratory animals (Scadding, 1980; Ratzlaff et al., 1992).

Exposure regimens and dosages

Two exposure regimens were used. In the first, the animals were treated by different routes with capsaicin at a 3µmole capsaicin/mouse dosage. The time between exposure and bleeding was 10 minutes. In the second regimen, for injection only, time spans between 5 minutes and 20 minutes post injection were examined for peak blood capsaicin concentrations, the injected dose being 3µmole capsaicin/ mouse. The last two time points, 15 and 20 minutes, did not produce capsaicin peaks on the HPLC integrator and were not repeated.

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Extraction of capsaicin from blood and tissue

Capsaicin levels in the blood were measured in a procedure modified from that described in the literature (Saria et al., 1981). Venous blood was collected from the animals at measured intervals after capsaicin exposure, and homogenized in ten volumes of ice cold acetone (Fisher) in a Dounce tissue grinder (Thomas Scientific, Swedesboro, NJ). Acetone extraction has the double advantage of precipitating most proteins and efficiently dissolving capsaicin. Acetone is also easily evaporated for purification purposes. The homogenate was placed into acetone resistant 15 ml polypropylene centrifuge tubes (Fisher), and centrifuged at 3000 x g in an IEC clinical centrifuge (Damon/IEC Division, Needham Heights, MA) for ten minutes. The supernatant fluid was collected and aliquoted into 1.5 ml volumes in 1.5 ml Eppendorf polypropylene micro test tubes, (Brinkmann Instruments, Inc., Westbury, NY). The acetone was then evaporated in a centrifugal evaporator under vacuum but without heating, using a Centrivac (Labconco Corporation, Kansas City, MO) to prevent bumping. The residue was re-dissolved in 1.5 ml acetone as above, centrifuged at 3000 x g for ten minutes, and the supernatant fluid was collected and dried in the Centrivac as described above. Finally, the sample was re-suspended in 150µl pure acetonitrile (Fisher). The samples were all tested within 1 week of preparation, as significant degradation occurs over time in capsaicin extracted from tissue samples.

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Measurement of capsaicin

The capsaicin levels in the samples were measured on a Waters model 441 (Waters Associates, Milford, MA) high pressure liquid chromatography (HPLC) machine. The chromatography conditions were as follows. The mobile phase was a degassed 60% acetonitrile/40% water solution, run at 1ml/minute and about 1795 pounds per square inch. The stationary phase (Column) was a reversed phase Altima 5 micron C_{18} of 250 x 4.5 mm size with a pore size of 100 Å (Altech). The column was equilibrated with the above solvent mixture for 1/2 hour before controls were run. The detector used was a Waters model 510 with a 214 nm filter. The resulting peaks were drawn by the attached Waters integrator and chart recorder, which also gave the area under the peaks. Control solutions of capsaicin in acetonitrile were run before and after the blood tissue samples to ensure the consistency of the peaks.

The area under the capsaicin peak for the control serial dilutions as determined by the Waters integrator was used to calculate the correlation between peak size and capsaicin concentrations. The linear regression obtained from the controls was used to estimate the amount of capsaicin in the mouse blood or tissue. Linear regression, and Analysis of Variance were performed by a computerized statistics package called Instat (Graphpad Inc., Charlottesville, VA), or by Microsoft Excel (Microsoft Corporation, Redmond, WA).

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RESULTS

EFFECT OF CAPSAICIN ON PRE-IMPLANTATION EMBRYO DEVELOPMENT

Early embryos were examined for developmental problems caused by capsaicin. These are evident in two ways. Abnormal development will result in unusual and/or uneven cell division. Second, there can be delayed development of the embryos between the two cell and the blastula stages. If capsaicin treated embryos showed either of these two traits, then it was concluded that there was a toxic effect that inhibited or otherwise altered embryo development.

Assay of ethanol controls and serial dilution

A preliminary study was performed to determine two factors. An assay was performed to determine the effect of adding 1% ethanol to the Krebs medium to the embryos. Second, embryos were co-cultured in a 10 fold serial dilution of capsaicin to obtain a general idea of capsaicin toxicity.

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Ethanol effects on embryo development

There was no significant difference between development of embryos grown in Krebs and development of embryos grown in Krebs + 1% ethanol. All embryos from both proceeded to blastocyst or hatching blastocyst by the fourth day. Thus, the values were identical by Chocran-Mantel-Haenszel (CMH) test of ranked data ($p = 0.99$). (Table 2)

Table 2
Comparison of embryo development in Krebs medium or Krebs + 1% ethanol.

Group:	Average Score *:	n = , p=
Krebs (Control)	Average Score = 4.8	n =21
Krebs + Ethanol	Average Score = 4.8	n =23, p=0.99

*Score based on developmental level at day 5: Degenerate embryo =0; 2-3 cell embryo = 1; 4-7 cell embryo = 2; 8-15 cell embryo = 3; morula = 4; blastocyst = 5.

Embryo development in Krebs with 1% ethanol and serial capsaicin concentrations

A ten fold serial dilution was made of the stock 300mM capsaicin/ethanol in order to obtain the following concentrations in Krebs + 1% ethanol: 3mM , 300μM , 30μM , 3μM , and 0.3μM. There was a significant difference between embryo development in the Krebs + ethanol and that in the Krebs + ethanol +

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3mM capsaicin ($p < 0.001$), as all of the embryos in this highest concentration capsaicin solution arrested development before entering the blastocyst stage. In fact, in this assay all of the embryos exposed to 3mM capsaicin degenerated by day 5. None of the other concentrations of capsaicin had any statistically significant effect on embryo development when compared with controls . The values were: for 300μM ($p= 0.560$); 30μM ($p= 0.337$); 3μM ($p= 0.560$); 0.3μM ($p= 0.586$)(Table 3)

Table 3
Comparison of embryo development in Krebs + 1% ethanol vs. serial concentrations of capsaicin in Krebs + 1% ethanol.

Group:	Average Score:	n =, p=
Control (+ Ethanol)	Score Av. = 4.4	n =16
+3mM Capsaicin	Score Av. = 0	n =16, $p < 0.001$
+300μM Capsaicin	Score Av. = 5	n =8, $p = 0.56$
+30μM Capsaicin	Score Av. = 4.4	n =7, $p = 0.337$
+3μM Capsaicin	Score Av. = 5	n =8, $p = 0.56$
+0.3μM Capsaicin	Score Av. = 5	n =7 $p = 0.59$

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Determination of the inhibitory concentration of capsaicin for embryo development

In the preliminary assays 3mM capsaicin prevented embryo development while 300μM capsaicin had no effect. Therefore, a series of concentrations between these two points were tested for inhibitory effect. The concentrations tested were 3mM , 2.2mM, 2mM, 1.5mM, 1.3mM, 1.1mM, and 1mM capsaicin. Of these, the 1mM and the 1.1mM concentrations did not significantly inhibit embryo development according to the statistical test (CMH, p values p= 0.694 and p= 0.937 respectively). For all of the other concentration, there was a definite statistically significant difference (by CMH test) in embryo development when compared to Krebs + 1% ethanol (in all cases, $p<0.001$). The approximate 50% effective dose (the dose that prevented maturation of 50% of the embryos) was between 1.3mM and 1.5mM capsaicin in Krebs + 1% ethanol. (Table 4)

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Table 4
Comparison of embryo development in Krebs + 1% ethanol vs. concentrations of capsaicin in Krebs + 1% ethanol.

Treatment	Average Score:	n = , p=
Control (+ Ethanol)	Score Av. = 5.0	n =71
3mM Capsaicin *	Score Av. = 1.7	n =40, p <0.001
2.2mM Capsaicin *	Score Av. = 0.69	n =23, p <0.001
2mM Capsaicin *	Score Av. = 2.07	n =14, p <0.001
1.5mM Capsaicin *	Score Av. = 2.6	n =15, p <0.001
1.3mM Capsaicin *	Score Av. = 3.25	n =16, p <0.001
1.1mM Capsaicin	Score Av. = 4.8	n =22, p =0.937
1mM Capsaicin	Score Av. = 4.7	n =15, p =0.694

*Capsaicin concentrations between 1.3mM and 3mM were toxic or inhibitory to embryo development.

EFFECT OF CAPSAICIN ON POST-IMPLANTATION FETAL DEVELOPMENT

It was anticipated that high doses of capsaicin may cause some bone or cartilage defects, any of which would be apparent on fetal clearing and staining. Abnormalities caused by capsaicin in bone or cartilage would be visible upon inspection under dissecting microscope. Capsaicin induced destruction of bone or cartilage would be evident in the fast growing bones of the feet or tail. Additionally, any inhibition of development would appear as a reduction in body length or body weight.

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Comparison of weight differences in fetuses treated with capsaicin, retinoic acid, or control

As the fetuses were removed from the dam on day 17, they were weighed. The measurements for the fetuses from each dam were averaged and the results of the averages from each treatment group were used for comparisons. A correction was applied for comparisons of fetus weights between series ($S_{corrected}$). The weight of each fetus was divided by the average weight of the control group for each series of assays, as was described in the methods. There was no significant difference between the weight of the fetuses in the groups treated with 3μmole and 0.3μmole capsaicin and the fetuses in the negative (vehicle treated) control ($p=0.42$ and $p = 0.86$ respectively). The positive control group embryos, however, were significantly heavier than the vehicle treated fetuses. The fetuses in the group treated with 1.6μM capsaicin were marginally significantly lower than the controls (by ANOVA comparison). It should be noted that only one dam in the 0.6μM capsaicin treated group survived and bore pups. This group was not used for any calculations, here or in any of the sections below. (Table 5)

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Table 5
Effect of capsaicin or retinoic acid treatment on fetal weight at day 17 PC.

Treatment	Average Weight, $S_{corrected}$	Standard Deviation	n =, p=
Vehicle	1	0.07	n =9, p=1.00
Positive Control	1.07	0.08	n =10, p=0.01*
3μmole Caps.	1.00	0.13	n =11, p=0.42
1.6μmole Caps.	0.87	0.15	n =5, p=0.05**
0.3μmole Caps.	1.02	0.01	n =2, p=0.86

* indicates Significance
**Indicates Marginal Significance

Comparison of length differences in fetuses treated with capsaicin, retinoic acid, or control

The average length, from snout to base of tail, was measured for the fetuses from all treatment groups (Figure 2). The difference in length of the positive control fetuses from that of the negative controls was marginally significant (p=0.04). Capsaicin did not appear to have any significant effect on fetal length in mice (p>0.05 for all cross comparisons). (Table 6)

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Table 6

Effect of capsaicin or retinoic acid treatment on fetus length at day 17 PC.

Treatment	Average Length, $S_{corrected}$	Standard Deviation	n =, p=
Vehicle	1	0.05	n =9, p=1.00
Positive Control	1.04	0.04	n =10, p=0.04**
3μmole Caps.	1.03	0.06	n =11, p=0.26
1.6μmole Caps.	0.96	0.08	n =5, p=0.29
0.3μmole Caps.	1.01	0.02	n =2, p=0.71

**Indicates Marginal Significance

Figure 2. Cleared 17 day mouse fetus. A = Humerus Length; B = Ossification; Blue = Cartilage; Red = Bone. Example of a typical cleared mouse fetus.

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Comparison of length of humerus vs. ossification differences in fetuses treated with capsaicin, retinoic acid, or control

The humerus on the cleared and stained fetuses was measured to determine degree of ossification. The humerus as a whole was measured, and the ossification of the humerus was measured (Figure 2). The ratio between the length of the humerus and the length of the ossification was obtained. The fetuses from each dam were averaged, and the averages for each treatment group were used for statistics. During fixation and staining, the fetuses from four of the dams were destroyed (2 positive controls, 1-0.6μmole capsaicin, and 1-0.3μmole capsaicin). Thus, they were not used in this part of the assay. There was no significant difference in the degree of ossification between the control (vehicle treated) mice and those treated with retinoic acid or any of the capsaicin concentrations ($p>0.05$ for all cross comparisons). (Table 7)

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Table 7
Effect of capsaicin or retinoic acid treatment on ossification of the humerus in the mouse fetus.

Treatment	Average Humerus Length/ Ossification	Standard Deviation	n =, p=
Vehicle	2.37	0.76	n =9, p=1.00
Positive Control	2.46	0.71	n =8, p=0.80
3μmole Caps.	2.35	0.74	n =10, p=0.57
1.6μmole Caps.	2.46	0.83	n =5, p=0.49
0.3μmole Caps.	3.34	0.23	n =2, p=0.12

Comparison of deformational differences in fetuses treated with capsaicin, retinoic acid, or control

The cleared and stained fetuses were examined for developmental defects as described in the methods. The deformities found can be described as follows. One fetus (#4) from mouse # 11, a retinoic acid treated mouse, had two tail vertebrae that were fused. One fetus (# 1) from mouse # 25, which was treated with 3μmole capsaicin, had two tail vertebrae that were fused. These two deformities do not amount to a significant difference between the capsaicin or retinoic acid treated mice and the control (vehicle treated) mice.

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BLOOD LEVELS OF CAPSAICIN AFTER EXPOSURE BY DIFFERENT ROUTES

It is evident from the literature (Saria et al., 1981; Saria et al., 1982) that capsaicin is capable of entering the bloodstream after ingestion. It was also thought that capsaicin will cross skin and mucous membranes. What remained to be seen is the amounts of capsaicin that actually enter the blood and thus the potential for neurological damage in unborn animals. It was anticipated that the amount of capsaicinoids that enter the bloodstream would be significant.

Standard curve for capsaicin measurement by HPLC

Using the area under the peak as supplied by the Waters integrator, a correlation was found between the peak length and the concentration of capsaicin injected.

The standard curve of the capsaicin concentrations did not perfectly match the theoretical curve. This is the result of two different factors. First, at low capsaicin concentrations, there is a significant interference from the absorption of the ultraviolet light (at 214 nm) by the solvent. Second, at the highest concentrations, there is an upper limit to which the detector will detect before it becomes saturated. The saturation of the detector may account for the break in the match of the standard curve at the highest concentration of

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capsaicin. The fit of the curve to the theoretical curve is nonetheless significant, with an R^2= 0.91. The formula for the curve is $Y=871.58e^{-1.5x}$. (Figure 3, also Appendix B)

Calculations of capsaicin levels in mouse blood after various treatments.

Using the standard curve, a theoretical concentration was obtained for the capsaicin in blood samples. Calculations for concentration were done by Instat, entering the peak values for each treatment as Y for a calculation of X according to the formula of the standard curve ($Y=871.58e^{-1.5x}$). Of the treatment groups, all the blood drawn from animals exposed to capsaicin, but not the blood drawn from animals injected with vehicle (without capsaicin), gave peaks on the HPLC integrator. However, none of the blood levels of capsaicin were significantly different from control levels. (Figure 4). The concentration for capsaicin in blood drawn from mice 10 minutes after SQ injection of 3µmole capsaicin was 0.027µM (Standard Deviation (SD)=$1.02x10^{-2}$, n =9; p = 0.21 compared to zero standard). Capsaicin levels in blood drawn from mice 5 minutes after SQ injection of 3µmole capsaicin was 0.006µM (SD=$5.2x10^{-3}$, p=0.16, n =6). Capsaicin levels in blood drawn from mice 10 minutes after oral ingestion of 3µmole capsaicin in oil was 0.006µM (SD=$2x10^{-3}$, p= 0.18, n =6). Finally, capsaicin levels in blood drawn from mice 10 minutes after topical application of 3µmole capsaicin in 89% DMSO /10% ethanol was 0.004µM. This latter point was not repeated, as the DMSO took a great deal of

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time to clear from the machine and there was fear of equipment damage. The zero control, that is blood drawn from mice not exposed to capsaicin, averaged a background peak of 0.001μM (SD=$5x10^{-3}$, n=9) None of these results were significantly different from zero, although they all showed clear peaks on the integrated graphs (Figure 4; also Appendix B). As was mentioned in the materials and methods, blood drawn from animals 15 minutes or more after SQ capsaicin injection did not produce a capsaicin peak on the HPLC integrator (Data not shown). This result is in accordance with evidence reported in the literature (Saria et al., 1982).

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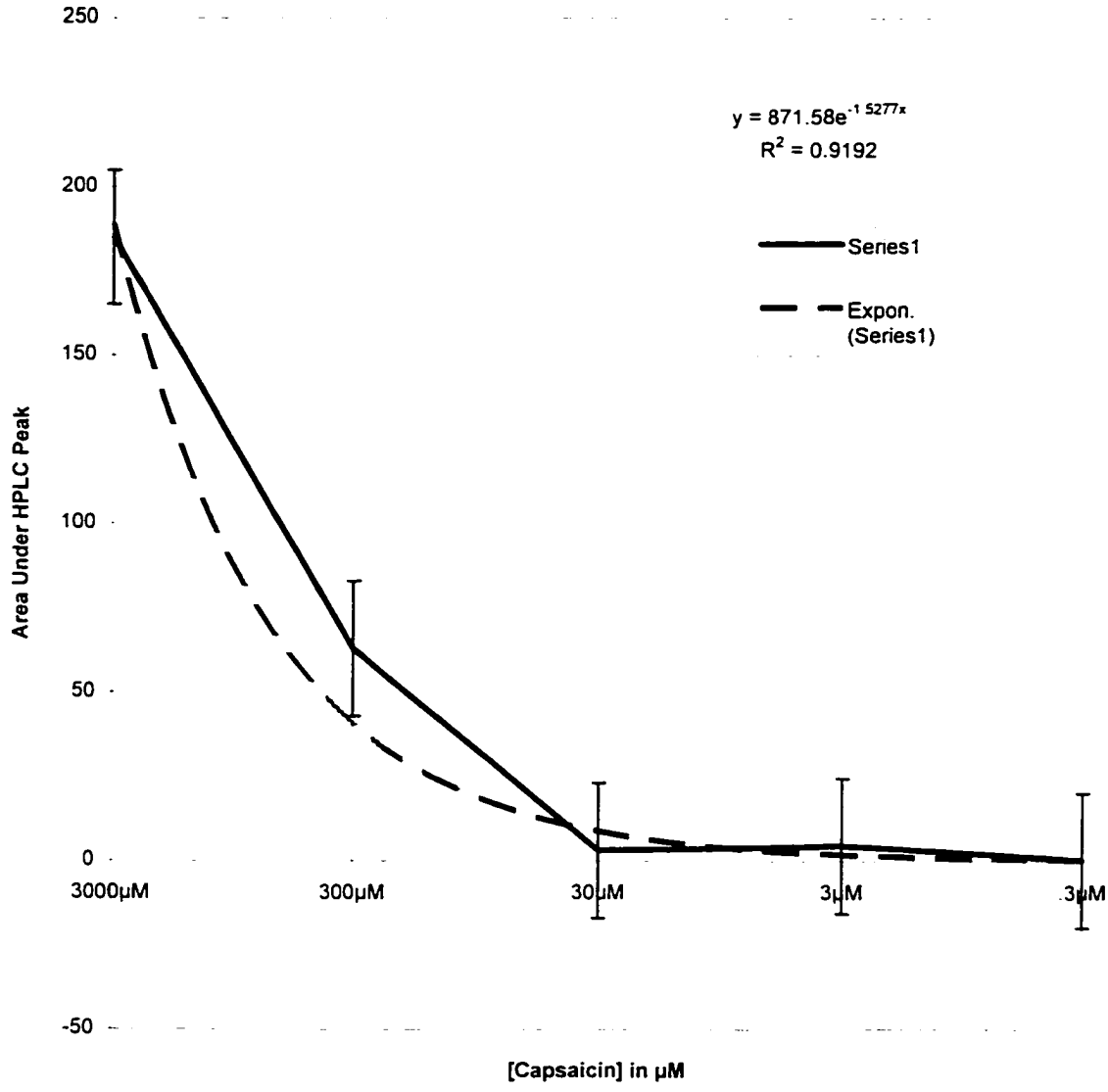

Figure 3. Standard curve of capsaicin concentration vs. area under HPLC peak (± SEM). Series 1 = standard curve. Expon (series 1) = theoretical curve.

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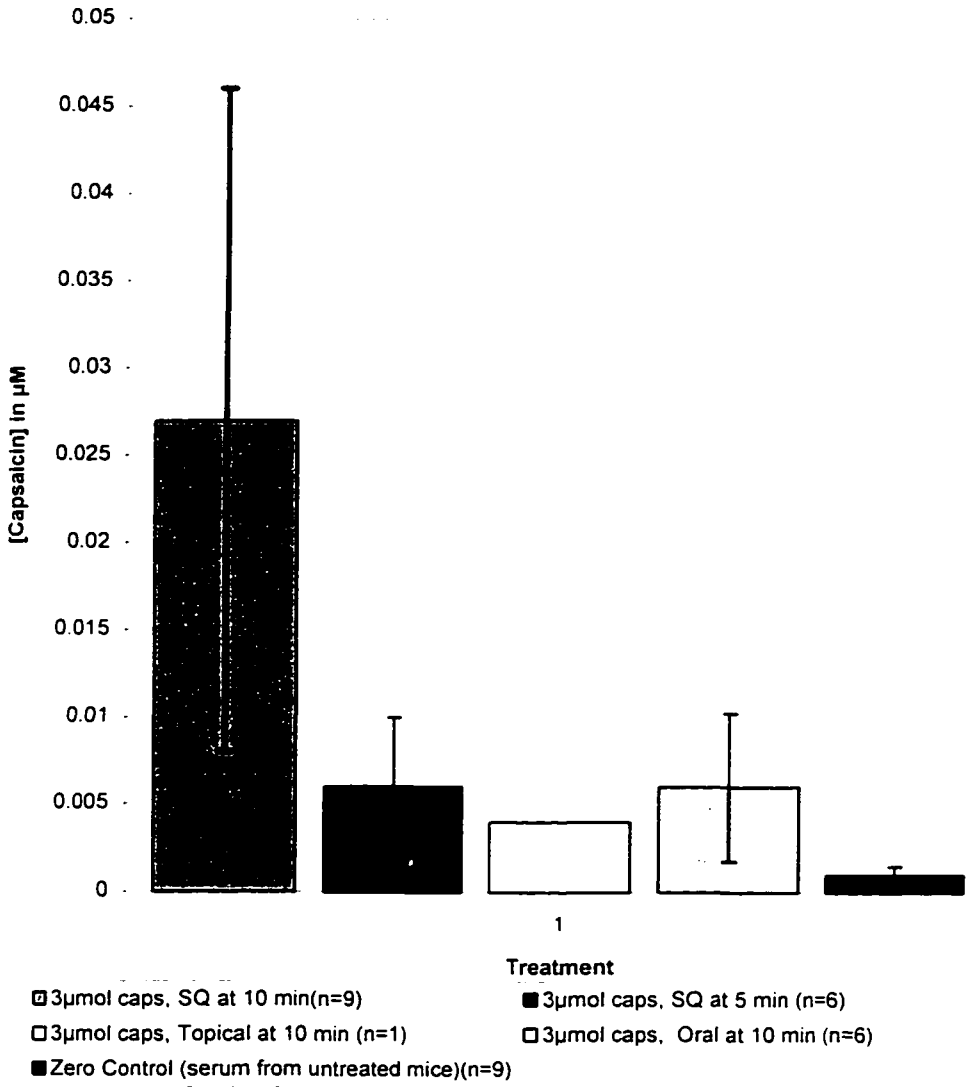

Figure 4. Concentrations of capsaicin in blood serum, after SQ injection, oral, and topical exposure (± SEM). Topical exposure n = 1, as described in the text. All exposures of 3µmol capsaicin, and all volumes 100 µl.

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DISCUSSION AND CONCLUSION

The *Capsicum* pepper has a long and colorful history (Lembeck, 1987; da la Vega, 1966). The use of this spice is widespread and the concentrations of pepper derived capsaicin can be quite high in food, medicines, and other preparations. Capsaicin is a potent neurotoxin with high specificity for C-afferent sensory nerve fibers. Mammals are particularly sensitive to capsaicin during the first several days post partum. Capsaicin causes a permanent loss of C-afferent fiber innervation in newborn mice and rats (Holzer, 1991). Capsaicin exposure and the accompanying loss of C-fiber innervation has been linked to reduction of immune response (Ratzlaff et al., 1992), loss of sensory perception (Lynn et al., 1992), and inhibition of several events associated with reproduction which result in impairment of fertility (Traurig et al., 1988; Nance et al., 1987: Traurig et al., 1984c).

Considering the widespread use and the neurotoxic potential of capsaicin, as well as the possibility of neurologic or developmental damage to fetuses, there is some concern regarding the possible contact of the fetuses with capsaicin, and of deleterious developmental effects of this agent. This research project addressed these questions. First, the direct effect on embryo development was measured through the use of an in vitro embryo co-culture assay. Second, the effect of capsaicin on post-implantation fetuses was examined using the embryo clearing method. Third, the degree of exposure

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was assayed by measuring the levels of capsaicin that reached the venous blood in exposed mice.

Capsaicin at high concentrations clearly interferes with pre-implantation embryo development. At 3mM and 1.5mM capsaicin levels, at least half of the embryos failed to develop. Even at levels of 1.3 mM capsaicin there was significant inhibition of development. These levels, however, are quite high compared to blood levels of capsaicin in animals treated to deplete their C-afferent fibers. The receptor mediated degranulation of C-afferent fibers is dose dependent, but degranulation of the neurons begins at concentrations in the nanomolar range (Caterina et al., 1997). In the embryo coculture assay described herein, capsaicin concentrations are in the millimolar range, or a million times more concentrated. The levels are such that it is unlikely that capsaicin interacts with receptors found on the embryo surface to produce these effects. It is much more likely that the capsaicin, which is lipophilic, is interacting directly with the cell membrane, perhaps interfering with membrane fluidity. This would have an effect on receptor function or on lipid signalling functions.

The levels of capsaicin in the blood of mice injected SQ with 3μmoles of capsaicin are at the lower limits of detection by the HPLC methods we used. It is known that 3μmoles (about 50-100mg/kg) capsaicin will cause degranulation of the C-afferent sensory nerve fibers in adult mice (Skadding, 1980; Holzer, 1991). By HPLC, however, the amount of capsaicin in the blood was not

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significantly different from control. Statistically, the amount of capsaicin in the blood could not be measured. This, of course, does not mean that there was no capsaicin in venous blood. There are several reasons why the capsaicin levels could not be measured. One of the problems was the variation of measured capsaicin levels between mice. The second is the low volume of blood that can be obtained from a mouse (about 0.3-0.5 ml). This volume does not lend itself readily to any form of concentrating techniques. Also, although capsaicin appears to be stable when in solid form or in ethanol or acetonitrile solution, we have found that when it comes in contact with blood or other tissue it is rapidly degraded. This is true even when the samples are maintained at -20° C.

Analysis of capsaicin levels in blood is of enough interest that it should be further explored. The problem remains that the levels of capsaicin in blood are very low. Capsaicin does not lend itself readily to many of the ultra-micro measurement detection techniques, such as immunoassays. At a molecular weight of 309 daltons, it is difficult to induce the production of monoclonal antibodies against this compound. A solution to the problem of microanalysis would be to possibly pool and then concentrate the capsaicin in the blood of several mice. A second way to solve the problem of variation in capsaicin blood levels and improve statistics is by using a very large population. Radiolabeled capsaicin might be measured in mouse blood, but this would have the disadvantage that inactive metabolites would be detected as well as the active

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compound, and radiolabeled capsaicin is not commercially available (Donnerer et al., 1990). The best solution awaits the development of better detectors. Perhaps fluorescent capsaicin analogs with better detection capabilities could be used (Lu, J., and Cwik, M., 1997). However, these analogs would most likely have quite different kinetics compared to the native capsaicin.

C-afferent sensory nerve fibers are thought to mediate the neurohormonal copulatory response critical for the formation of the deciduum (Traurig et al., 1988). Embryos are dependent on the decidual response for completion of implantation and maturation. Further, SP has been shown to be capable of indirectly affecting the release of LH and FSH by increasing the release of GnRH (Kordon et al., 1994). Finally, it is known that capsaicin will penetrate the uterus and the blood placenta barrier and may cause neurologic damage to fetal rats (Pellicer et al., 1996). Days 8, 9, and 10 are critical to the development not only of cartilage and bone, but also of nerves and internal organs (Hogan, 1986). Thus, it could be expected that capsaicin would have an effect on late embryo/ early fetal development due to effects on either the fetus or the dam. This is not what was found. Capsaicin had no effect on the bone and cartilage development in post-implantation fetuses. There was no effect on the length or the weight of the developing fetuses. The dosages injected (100ul of 33mM (1%) capsaicin) were high enough to cause degranulation of the C-afferent fibers in the dam as well as the fetuses (Pellicer et al., 1996; Green 1966). It is apparent, therefore, that the post-implantation

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fetus and the dam are less dependent on the neurohormonal complex involving the C-afferent fibers than are the pre-implantation embryos (Traurig et al., 1988). If capsaicin-sensitive C-afferent fibers are involved in the decidual response, then once the decidual response has taken place they are less critical to fetal development (Flietstra and Voogt, 1997).

The levels of capsaicin that are effective in inhibiting embryo development in the mouse co-culture assays were quite high, especially when compared to the levels which are likely to be found in the blood stream, even under the most extreme conditions. By the time the blood reaches the uterus and fallopian tubes where the periimplantation embryos are found, capsaicin levels are much lower. Thus, it is unlikely that capsaicin exposure by any route including injection will directly affect pre-implantation embryos. Further, there are no detrimental effects of capsaicin exposure to limb formation in the post-implantation embryo. Capsaicin can have an effect on embryo development in two different ways. First, it has been reported to inhibit the decidual cell response (Traurig et al., 1988). This will affect implantation and therefore fecundity, but will not necessarily affect the normal development of the fetuses that succeed in implanting successfully. Second, it is very likely that there is significant neurological damage to the fetus (Pellicer et al., 1996). If this is indeed the case, then the C-afferent fibers do not play an obvious role in the development of cartilage and bone, or in the correct functioning of the structures associated with gestation. It can therefore be concluded that in mice, any

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embryo that successfully implants and is carried to term will be born normal in appearance if not in behavior or physiology. If the mouse embryo development is used as a model for embryo development in humans, then the conclusions one can draw from this study is that there is little danger of birth defects from capsaicin exposure.

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FUTURE STUDIES

There are several questions that remain to be answered. First, the evidence for a capsaicin effect on the implantation is indirect and stems from two factors: the decrease in fecundity in female mice treated with capsaicin as newborns, and the capsaicin-induced loss of pseudo-pregnancy initiated by vaginal stimulation (Traurig et al., 1988; Nance et al., 1987; Traurig et al., 1984b). Vaginal/cervical-stimulation is critical for the successful formation of the deciduum (Green, 1966; Hogan, 1986; Nance et al., 1987). However, capsaicin effects on implantation can be tested directly by embryo transfer (for a reference, see Betteridge and Rieger, 1993), and the decidual reaction can be studied by artificially irritating the uterus. A more direct evaluation of the capsaicin effect on the decidual response is therefore possible.

Capsaicin levels in the embryo and the reproductive structures need to be obtained successfully. The measurement of these levels was not possible under the conditions tested. This data will be of great help in determining the possible effects of capsaicin exposure on the embryo. Again, indirect evidence suggests that capsaicin easily passes the blood-placenta barrier. This needs to be measured directly.

Neurological damage to the fetus was not assessed. This study was focused on reproductive structures and visible fetus defects. However, neurological damage is not necessarily linked to visible damage. A study of the

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induction of neurological damage by capsaicin treatment at various times during gestation would pinpoint the critical times for development of the C-afferent fibers or their precursors in the mouse embryo. Detection of neurological damage could perhaps be done by immunocytochemical methods, using SP as a marker for C-afferent fibers. The receptor for capsaicin has recently been discovered (Caterina et al., 1997). The appearance of this receptor during development could perhaps be detected, again by immunocytochemistry.

Capsaicin has been shown to open sodium-calcium ion channels. There are several events that take place during oogenesis and embryogenesis that are driven by calcium or other ion fluxes in the egg. One that is of particular interest is the cortical granule reaction that in turn induces the zonal reaction and prevents sperm penetration. If capsaicin indeed affects the calcium channels in the unfertilized egg, it could either trigger or inhibit the cortical granule reaction. Either effect would have a negative effect on fecundity. Inhibition of the reaction could result in failure to initiate the zonal reaction and thus increase the possibility of polyspermy. A spontaneous induction of the cortical granule reaction would prevent fertilization. In either case, a successful fertilization would be prevented.

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APPENDIX A

Table A-1. Krebs -Ringer's Bicarbonate Media. (Ackerman and Swanson, 1983)

Solute	Concentration
NaCl	99.6mM
Kcl	4.78mM
$CaCl_2$	1.71mM
$MgSO_4$	1.19mM
Na HCO_3	1.19mM
Na Lactate	21.58mM
Na Pyruvate	0.50mM
Glucose	5.56mM
Bovine Serum Albumin	4.0 mg/ml
Penicillin	50 I.U./ml
Streptomycin	50 µg/ml

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APPENDIX B

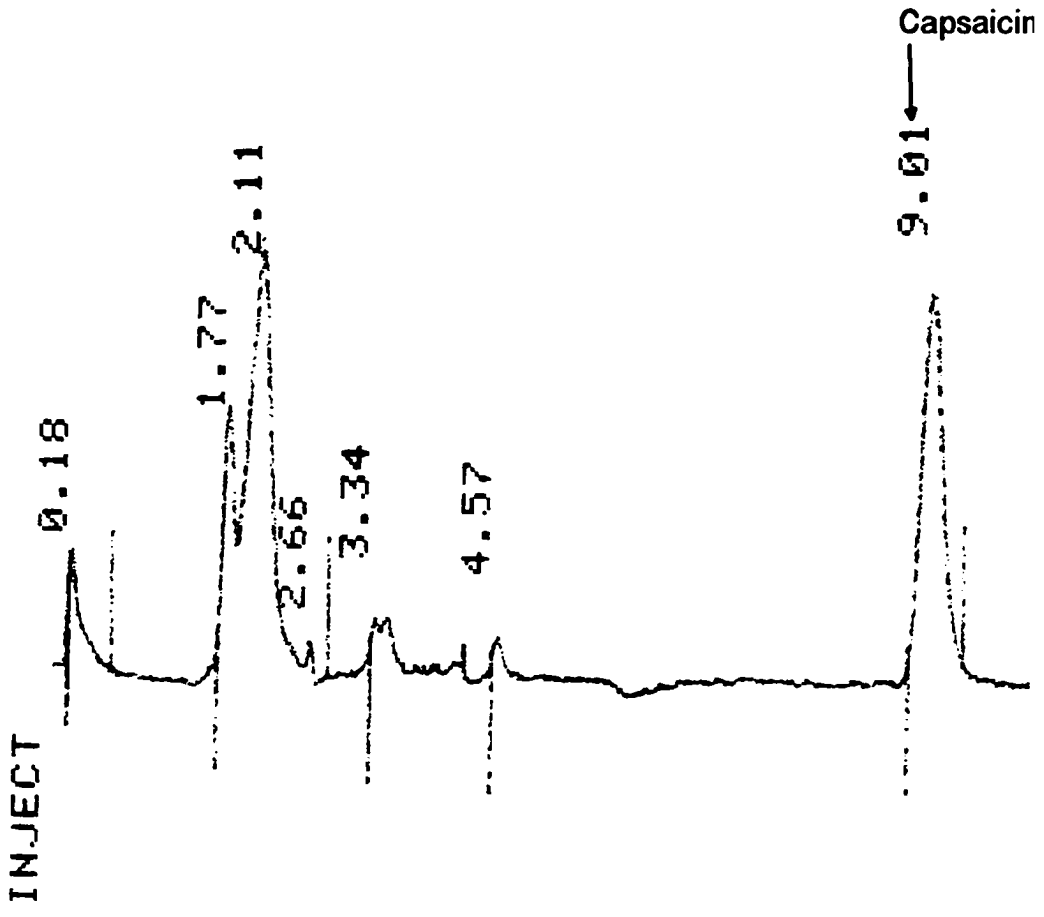

Figure A-1. HPLC chart of standard : injection of 10µl of 0.3µM capsaicin in acetonitrile. Capsaicin peak at approximate timepoint of 9 minutes.

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Figure A-2. Representative experimental HPLC peak: 10µl of acetone extract of serum from mouse injected with 3µmol capsaicin. Time elapsed between injection and extraction 10 minutes. Capsaicin peak at approximate timepoint of 9 minutes.

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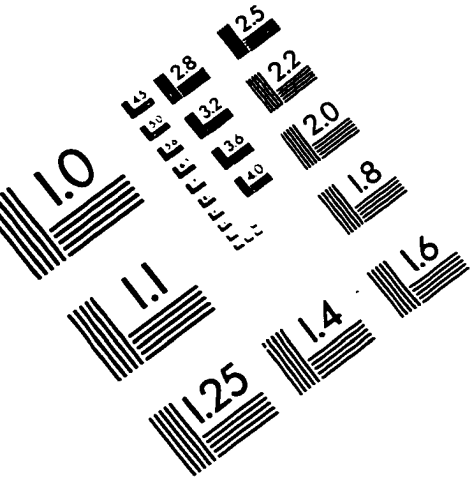
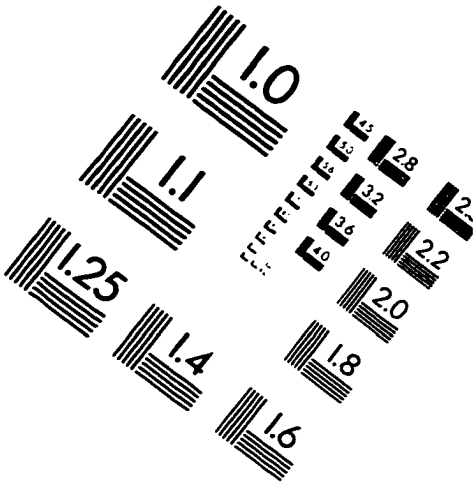
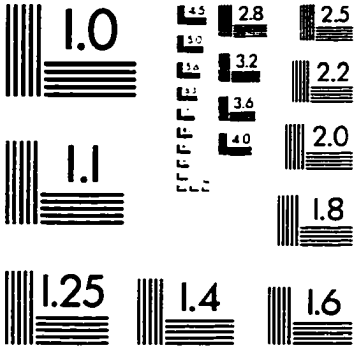
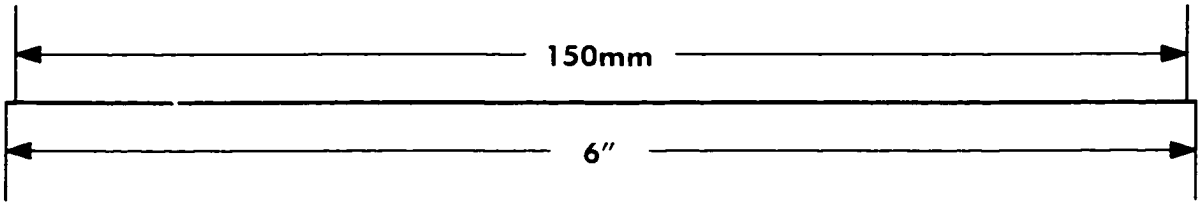
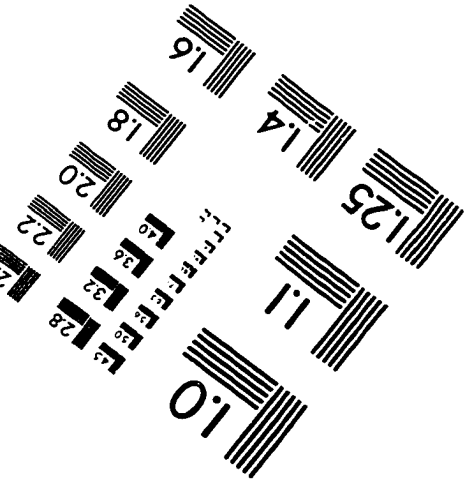
IMAGE EVALUATION TEST TARGET (QA-3)

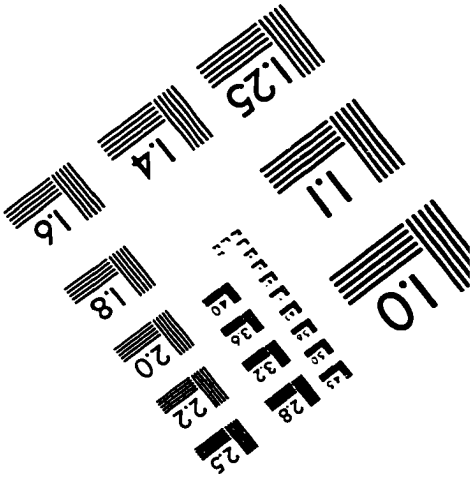












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