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A Novel Method for Assessing Sex-Specific and Genotype-Specific Response to Injury in Astrocyte Culture

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Abstract

Female astrocytes sustain less cell death from oxygen-glucose deprivation (OGD) than male astrocytes. Arimidex, an aromatase inhibitor, abolishes these sex differences. To verify sex-dependent differences in P450 aromatase function in astrocyte cell death following OGD, we developed a novel method that uses sex-specific and genotype-specific single pup primary astrocyte cultures from wild-type (WT) and aromatase-knockout (ArKO) mice. After determining sex by external and internal examination as well as PCR and genotype by PCR amplification of tail cDNA, we established cultures from 1−3 day-old male and female, WT and ArKO mice pups and grew them to confluence in estrogen-free media. Cell death was measured by lactate dehydrogenase (LDH) assay. Our study shows that, while WT female astrocytes are more resistant to OGD than WT male cells, sex differences disappear in ArKO cells. Cell death is significantly increased in ArKO compared to WT in female astrocytes but not male cells. Therefore, P450 aromatase appears to be essential in endogenous neuroprotection in females, and this finding may have clinical implications. This innovative technique may also be applied to other *in vitro* studies of sex-related functional differences.

Keywords

aromatase; sex; astrocyte; cell death; neuroprotection

1. Introduction

Stroke is a sexually dimorphic disease. In humans, for example, male and female differences in stroke risk and outcome have been reported (Holroyd-Leduc, et al., 2000; Di, et al., 2003; Kapral, et al., 2005). Female sex is associated with favorable outcome from ischemic stroke compared to male animals, and these sex differences are attributed in part to the protective effect of estrogen (Alkayed, et al., 2000; Murphy, et al., 2004).

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In brain, estrogen can be synthesized locally from testosterone via P450 aromatase. Female mice with targeted deletion of P450 aromatase (encoded by *cyp19*) sustained increased brain damage after middle cerebral artery occlusion compared to wild-type (WT) female littermates or even ovariectomized females (McCullough, et al., 2003). Accordingly, brain aromatase may be important to ischemic pathophysiology. Consistent with this hypothesis, aromatase activity is induced in astrocytes after brain injury (Garcia-Segura, et al., 1999; Azcoitia, et al., 2003).

We recently reported that astrocytes isolated from neonatal cortex exhibit marked sex differences in sensitivity to oxygen-glucose deprivation (OGD) in part due to higher aromatase expression in female astrocytes compared to male cells. The aromatase inhibitor Arimidex abolished sex differences in OGD-induced cell death (Liu, et al., 2007). In this study, we developed a method to establish an *in vitro* ischemic model using P450 aromatase-knockout (ArKO) mice generated from heterozygous breeding harems. We grew sex-specific and genotype-specific WT and ArKO single pup primary astrocyte cultures to verify the neuroprotective effect of P450 aromatase in astrocytes following OGD. By applying this new method, we tested the hypothesis that P450 aromatase plays a key role in mediating the sex difference in astrocyte survival, and we demonstrated that P450 aromatase gene deletion abolishes the sex difference in astrocyte cell death.

In contrast to previous *in vitro* methods, our novel technique uses cultures that are both sex-specific and genotype-specific to study molecular mechanisms of cell death and cell survival that will lead to new therapeutic targets and improved outcomes following stroke and other neurologic diseases.

2. Materials and Methods

All animal procedures were conducted in accordance with the National Institutes of Health guidelines for care and use of animals in research, and protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

2.1. Sex determination

Astrocytes were cultured from 1−3 day-old single male and female Sprague-Dawley pups separately (dams from Charles River, Wilmington, MA). Male and female rat pups were distinguished by a larger genital papilla and longer ano-genital distance in male vs. female pups. Sex was confirmed by inspecting internal organs and gonads after laporatomy, e.g., uterine horn in females and testis in males, and by multiplex polymerase chain reaction (PCR) using male-specific marker SRY (sex determination region on the Y chromosome responsible for testes formation) and the universal marker myogenin (Myog) (expressed in both males and females) as previously described (McClive and Sinclair, 2001).

2.2. P450 aromatase-deficient single pup genotyping by PCR

P450 aromatase-knockout ($Ar^{-/-}$, ArKO) mice were generated as previously described (Fisher, et al., 1998). This strain originated on a C57BL/6J;129SvEv background and was backcrossed to C57BL/6J for at least 10 generations. In ArKO mice, exon IX of the *Cyp* 19 gene is disrupted by inserting a neo cassette using homologous recombination. Homozygous ($Ar^{-/-}$) mice produce an abnormal transcript that generates a nonfunctional protein. Consequently, aromatase activity is lacking in all cell types, and estrogen synthesis is blocked. $Ar^{+/-}$ (heterozygote) breeder pairs were obtained from Dr. Orhan K. Oz, Department of Radiology, University of Texas Southwestern Medical Center. Female ArKO ($Ar^{-/-}$) mice are infertile due to arrested folliculogenesis (Findlay, et al., 2001), and ArKO ($Ar^{-/-}$) males are hypofertile due to impaired sexual behavior and an age-dependent disruption of spermatogenesis (Murata,

et al., 2002). Therefore, the breeding colony was maintained with heterozygous ($Ar^{+/-}$) breeding harems (2 females, 1 male).

Genotype was determined using PCR amplification of tail DNA, as previously described (Robertson, et al., 1999; Britt, et al., 2000), to identify exon IX and the neo insert in the *Cyp* 19 gene. Offspring were screened with two sets of primers. The first primer pair (5' GTG ACA GAG ACA TAA AGA TCG 3' and 5' GTA AAT TCA TTG GGC TTA GGG 3') produced a 220 base pair (bp) product for the WT allele and no product for the ArKO allele. The second primer pair (5' ATC AGG ATG ATC TGG ACG AAG A 3' and 5' CCA CAG TCG ATG AAT CCA GAA 3') produced a 170 bp product for the ArKO allele and no product for the WT allele. The PCR products were resolved by electrophoresis in 2.5% agarose gel and were visualized by ethidium bromide staining. The expected sizes of the PCR products are 220 bp in $Ar^{+/+}$ (WT) and 170 bp in $Ar^{-/-}$ (ArKO); and both PCR products are present in $Ar^{+/-}$ (heterozygote). Every set of PCR reactions included a negative control (no DNA) and a positive control (heterozygous DNA).

2.3. Sex-specific and genotype-specific single pup primary astrocyte cultures

After segregation of male and female WT and ArKO mice using the methods described above, primary cultures of single pup male and female cortical astrocytes were prepared seperately, as previously published (Liu and Alkayed, 2005). The cell suspension was diluted with a feeding media consisting of 10% charcoal-stripped, estrogen-free fetal bovine serum (HyClone, Logan, Utah) in DMEM (Invitrogen) with 1% penicillin-streptomycin. The cells were seeded in 24-well plates for cell death assay and incubated at 37°C in 95% / 5% mixture of atmospheric air and $CO_{2.}$. Feeding media were changed after 2 days and subsequently twice a week. Confluent monolayers [10 to 14 days *in vitro* (div)] of primary cortical cultures consisting of 98.4 ± 0.5% glial fibrillary acidic protein (GFAP)-positive cells were used, as previously described (Liu and Alkayed, 2005).

2.4. Oxygen-glucose deprivation (OGD) and cell death assay

At 10−14 div, the feeding media were replaced with oxygen-depleted, glucose-, serum-and phenol red-free DMEM, and the cultures were incubated for 12 hours in a Coy™ anoxia chamber filled with an anoxic gas mixture containing 5%H2/5%CO2/90%N2 at 37°C. Anoxic conditions were monitored using an oxygen sensor that maintained oxygen levels at 0 p.p.m (Coy Laboratories Products, Grass Lake, MI, USA). Immediately after OGD, the media were replaced with fresh feeding media containing glucose, and the cells were returned to normoxia for 24 hours. Cell death was measured by lactate dehydrogenase (LDH) release into the culture medium (LDH cytotoxicity detection kit, Roche). Experiments were repeated on 12 independent cultures from different litters, and data was averaged from 3 wells per condition per experiment to n = 1.

2.5. Statistical analysis

Cells prepared from single male vs. female pup constitute one experiment (n = 1). The number of experiments (n) for any sex refers to number of cultures prepared from different litters. Differences between male and female astrocytes were determined by ANOVA with *post hoc* Newman Keuls. Values are presented as mean ± SEM with statistical significance at $p < 0.05$.

3. Results

3.1. Sex determination

Visual sex identification (Fig. 1A and Fig. 1B) was highly reliable (100% accuracy, n = 10), as verified by subsequent PCR confirmation of sexual genotype (Fig. 2A). Male and female

rat pups were distinguished by a larger genital papilla and longer ano-genital distance in male vs. female pups (Fig.1A). Sex was confirmed by inspecting internal organs and gonads after laporatomy, e.g., uterine horn in females and testis in males (Fig. 1B). Sex was determined by PCR using the male-specific marker SRY (sex determination region on the Y chromosome, responsible for testes formation) and the universal marker myogenin (Myog), which is expressed in both males and females. Female pups express Myog gene only, and male pups express both SRY gene and Myog gene (Fig. 2A).

3.2. Genotyping by PCR

Fig. 2B illustrates the single pup PCR genotyping for tail DNA analysis. As expected, the PCR products were 220 bp for $Ar^{+/+}$ (WT) and 170 bp for $Ar^{-/-}$ (ArKO). Both PCR products were present for $Ar^{+/-}$ (heterozygote, HT).

3.3. Response of sex-specific and genotype–specific ArKO and WT single pup primary astrocyte cultures to OGD

When subjected to 12-hour OGD, WT female astrocytes were more resistant to OGD than WT male cells. Cell death at 24 hours was 25.9±1.3% (n = 12) in female and 43.7±2.1% (n = 12) in male (Fig. 3) ($p < 0.05$).

To determine if differences in aromatase activity mediate the sex-specific response to OGD, we used aromatase-knockout mice (ArKO). Cell death markedly increased following OGD in ArKO female astrocytes compared to WT female cells, but not in ArKO male astrocytes compared to WT male cells. Therefore, aromatase-deficiency abolished sex-specific responses to OGD (48.2±2.5%, n = 12 in F vs. 49.1±2.6%, n = 12 in M, $p > 0.05$) (Fig. 3).

Discussion

Using our new sex-specific, genotype-specific method for culturing single pup primary astrocytes to achieve an *in vitro* ischemic model, we found that sex-specific responses to OGD can be altered by genotype. We also observed for the first time that female but not male astrocytes are dependent on the integrity of the aromatase gene.

There are gender differences in many diseases. For example, stroke is a sexually dimorphic disease, starting from male and female differences in stroke incidence and outcome in humans, to experimental studies that clearly show differences in ischemic sensitivity between male and female animals. This sex difference in ischemic brain injury persisted in genetic *in vivo* models of hypertension (Alkayed, et al., 1998) and diabetes (Hurn and Macrae, 2000; Toung, et al., 2000; Vannucci, et al., 2001; Sieber, et al., 2001), suggesting that fundamental molecular mechanisms of ischemic cell death may be gender dependent. These differences are in part due to the protective effect of the female sex hormone estrogen (Hurn and Macrae, 2000; McCullough, et al., 2003; Liu, et al., 2004), since ovariectomy increases ischemic brain damage in female rats (Alkayed, et al., 1998) and mice (McCullough, et al., 2005), and estrogen replacement is protective against cerebral ischemia in ovariectomized rats (Rusa, et al., 1999) and mice (McCullough, et al., 2005) and in reproductively senescent male and female rats (Alkayed, et al., 2000).

In recent *in vitro* studies, neuronal survival was different in male vs. female brain cells despite similar culture media composition (Zhang, et al., 2003). XX vs. XY cells respond differently to simulated ischemia and toxicity. For example, XY neurons are more susceptible to glutamate or peroxynitrite exposure than are XX cells, whereas XX cells are more sensitive to proapoptotic stimuli (Du, et al., 2004). We reported that astrocytes isolated from neonatal cortex exhibited marked sex differences in sensitivity to oxygen glucose deprivation (OGD)

in part due to higher aromatase expression in female astrocytes compared to male cells. The aromatase inhibitor Arimidex abolished sex differences in OGD-induced cell death (Liu, et al., 2007), suggesting that sex differences in ischemic cell death are related to aromatase expression (Liu, et al., 2007). Such experiments conducted in steroid-free media indicate that some mechanisms of injury (or survival) may be linked to the genetic sex of the cell.

In this study, we further delineated our sex-specific *in vitro* ischemic model by using aromatase-knockout (ArKO) mice to develop single pup primary astrocyte cultures that are also genotype-specific. We bred these ArKO mice from heterozygous colonies, since female $ArKO^{-/-}$ mice are infertile due to arrested folliculogenesis (Findlay, et al., 2001) and $ArKO^{-/-}$ males are hypofertile due to impaired sexual behavior and an age-dependent disruption of spermatogenesis (Murata, et al., 2002). Three types of pups were born: wild-type (WT), knockout (ArKO), and heterozygote (HT). In agreement with data generated in sex-specific rat astrocytes (Liu, et al., 2007), we found that female WT mouse astrocytes are more resistant to oxygen-glucose deprivation (OGD) than male WT mouse astrocytes. It is important to note that male sensitivity and female resistance to OGD are observed across species. Using the new technique developed for this study, we demonstrated that P450 aromatase gene deletion significantly increased cell death after OGD in female astrocytes and abolished the sex differences in sensitivity to OGD seen in WT female and male astrocytes. These findings, then, confirm that P450 aromatase is instrumental in mediating astrocyte survival following OGD and suggest that P450 aromatase is neuroprotective against ischemic brain injury. This is consistent with an earlier *in vivo* study of ischemia in which brain damage was greater in female ArKO mice compared to wild-type (WT) female mice (McCullough, et al., 2003).

Cell response to injury *in vitro* can be sex-specific. When gathering preclinical data, therefore, it is crucial to use sex-specific cell cultures to characterize these mechanisms and to avoid erroneous conclusions, as clinical trials of new therapies and drugs require representation of both sexes. Pharmacological inhibition or gene deletion may be important to further delineate sex-specific molecular mechanisms. This study is the first to combine sex-specific and genotype-specific techniques to investigate cell death and cell survival pathways following ischemia in single pup primary astrocyte cell cultures.

In conclusion, results of this study indicate that the biosynthetic enzyme P450 aromatase has neuroprotective effects in females and may have therapeutic implications for clinical stroke. These findings underscore the importance of studying male and female cells separately and in genotype-specific cultures to better understand sex-specific pathways of cell death and survival following ischemia.

This sex-specific, genotype-specific *in vitro* method can also be a very useful tool to improve accuracy and reduce interpretation errors in other studies on sex-related differences in molecular mechanisms.

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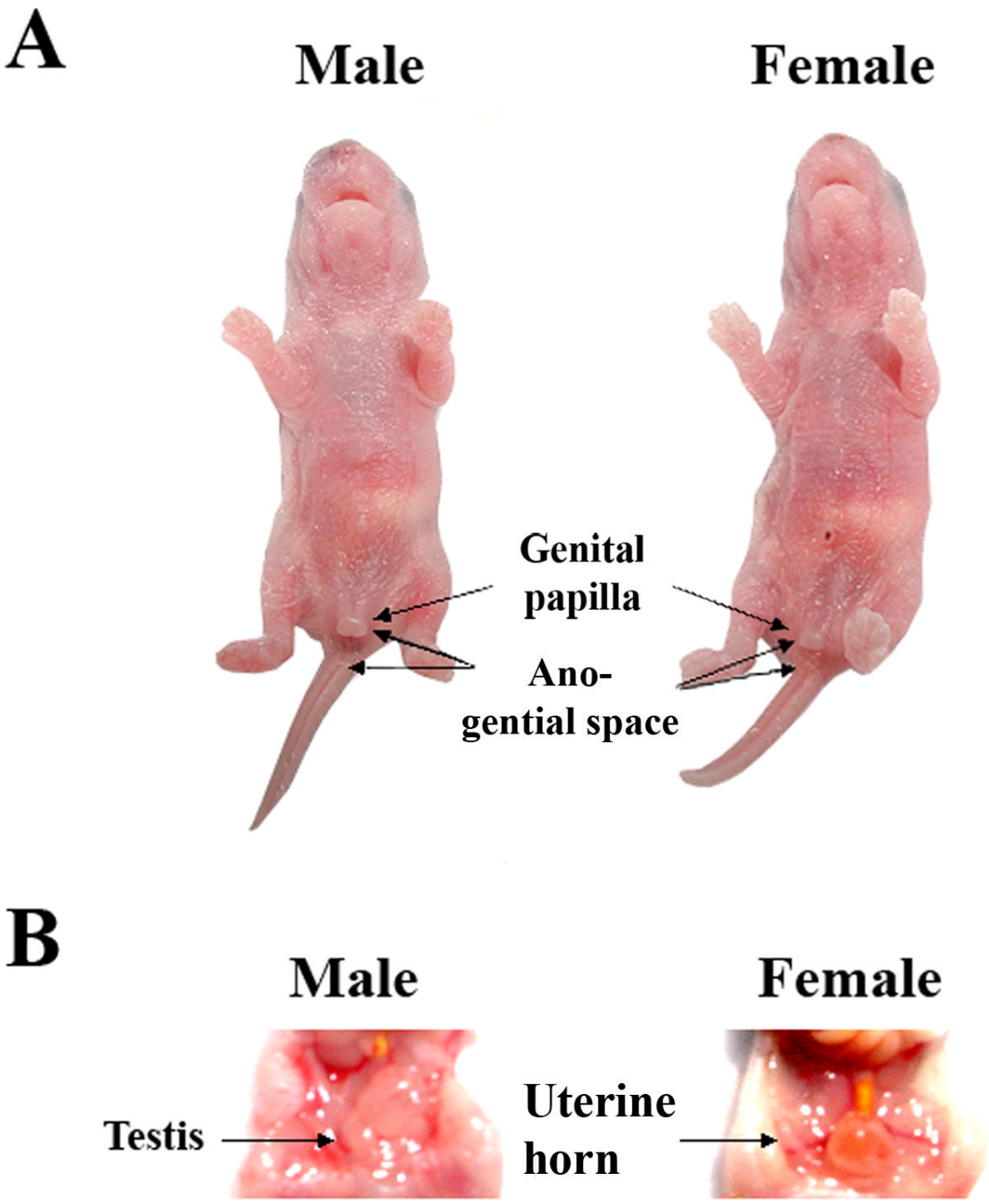

Fig. 1A.
Male and female rat pups were distinguished by a larger genital papilla and longer ano-genital distance in male vs. female pups.
Fig. 1B. Sex was confirmed by inspecting internal organs and gonads after laporatomy, e.g., uterine horn in females and testis in males.

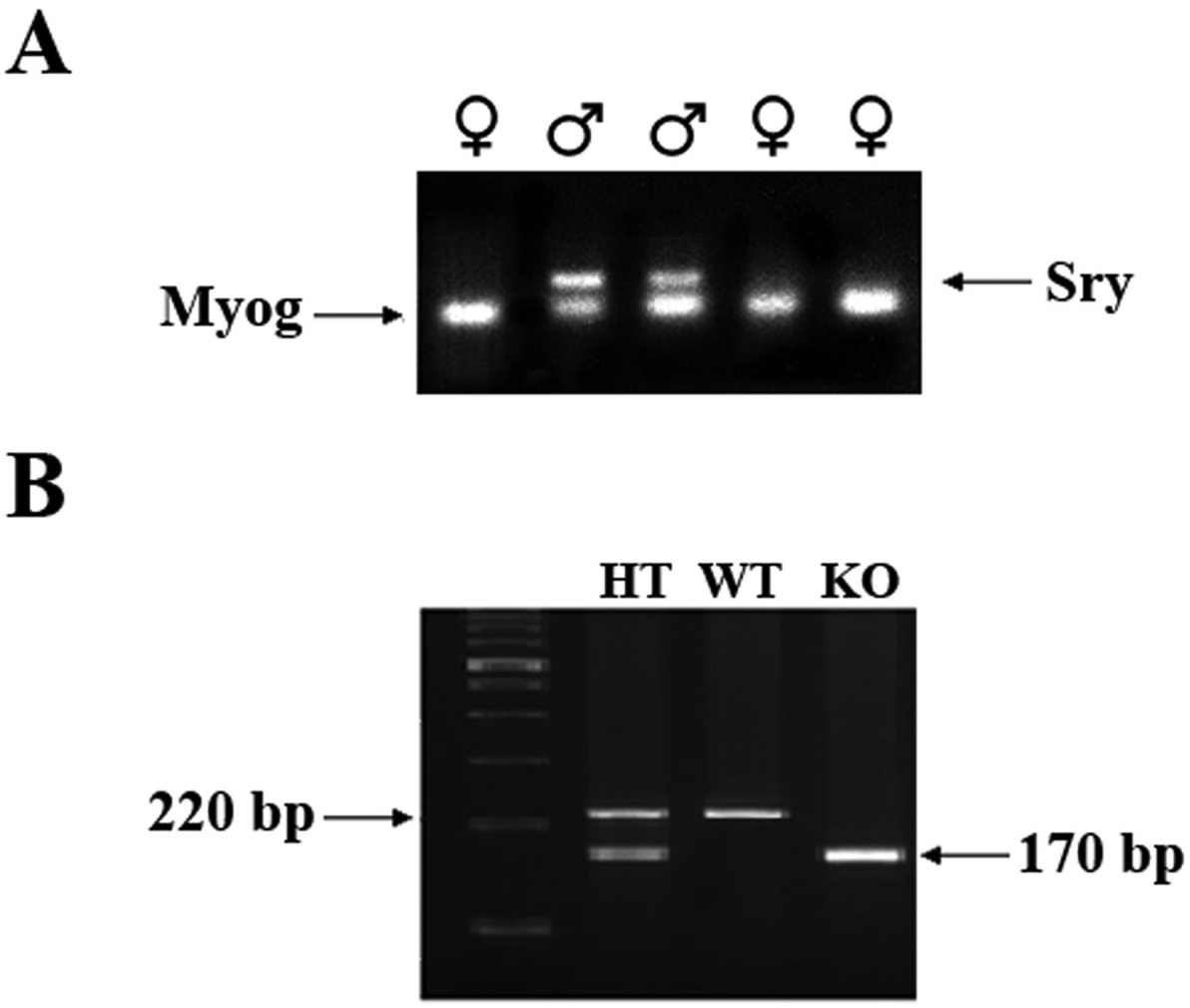

Fig. 2A.
Sex was determined by PCR using the male-specific marker SRY (appears in the sex determination region on the Y chromosome and is responsible for testes formation) and the universal marker myogenin (Myog) (expressed in both males and females). ♀ pups express Myog gene only, ♂ pups express both SRY gene and Myog gene.
Fig. 2B. PCR genotyping for tail DNA, the PCR products are 220 base pairs (bp) for $Ar^{+/+}$ (WT), 170 bp for $Ar^{-/-}$ (ArKO), and both of these PCR products for $Ar^{+/-}$(heterozygote, HT).

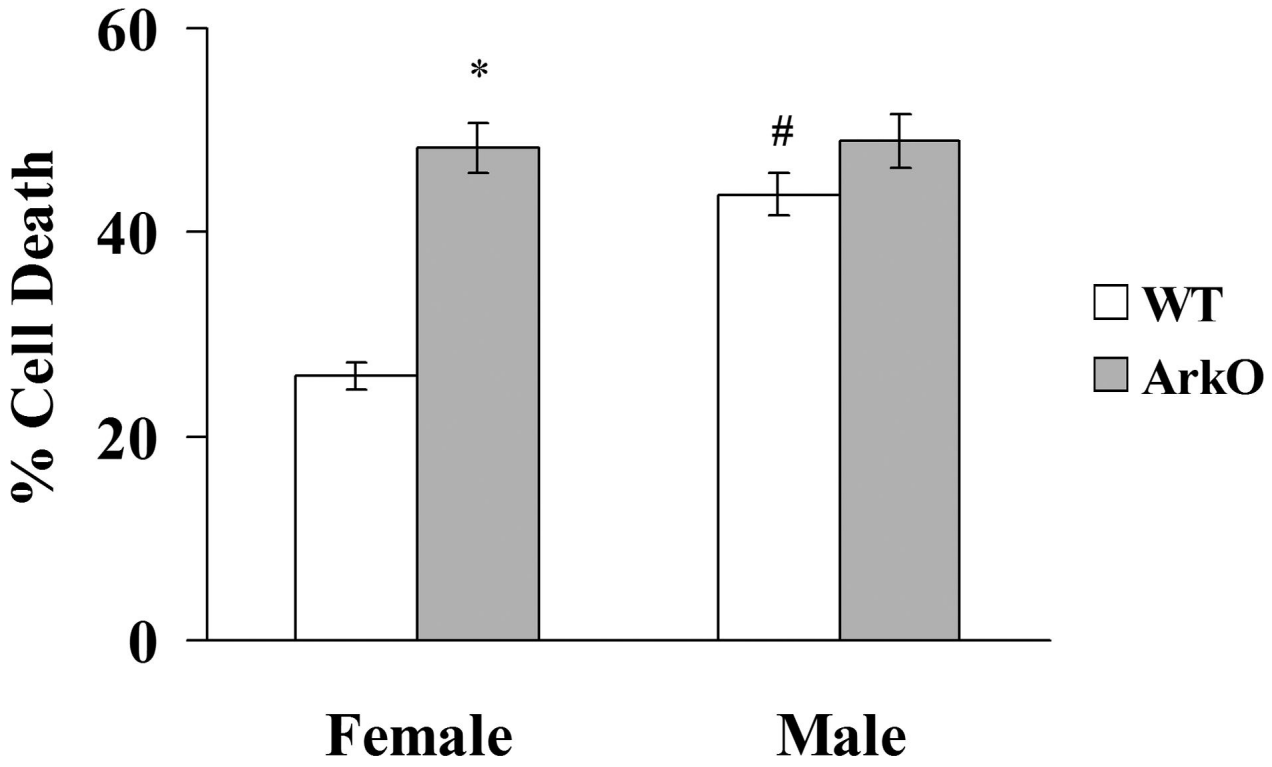

Fig. 3.

WT female astrocytes are less susceptible to cell death compared to WT male astrocytes after OGD (n = 12, $p < 0.05$, # - Significant difference in WT female astrocytes compared to WT male cells). Cell death markedly increased in ArKO female astrocytes compared to WT female cells, but there was no significant difference in cell death between ArKO male astrocytes compared to WT male cells. Sex-specific responses to OGD were abolished in ArKO astrocyte cultures, (n = 12, $p < 0.05$, * - Significant difference in ArKO female astrocytes compared to WT female cells).