The neuroendocrine effects of dehydroepiandrosterone and 17β-estradiol in the *in vitro* preterm hyperoxia infant model

Stephanie Hübnner, Donna E. Sunny, Christine Zäadow, Johanna Ruhnau, Bettina Reich, Antje Vogelgesang, Matthias Heckmann

1 Department of Neonatology and Pediatric Intensive Care, University Medicine Greifswald, 17475 Greifswald, Germany
2 Department of Neurology, University Medicine Greifswald, 17475 Greifswald, Germany
3 Department of Congenital Heart Disease and Pediatric Cardiology, German Heart Center Munich, Technical University of Munich, 80636 Munich, Germany

*Correspondence: matthias.heckmann@uni-greifswald.de* (Matthias Heckmann)

These authors contributed equally.

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Preterm birth causes neurological deficits. Previously, we demonstrated that fetal zone steroids reduce hyperoxia-mediated cell death *in vitro*. In immature oligodendrocytes (OLN-93 cells), dehydroepiandrosterone + 17β-estradiol co-treatment had synergistic beneficial effects while signals were transduced through different receptors. In immature astrocytes (C6 cells), both hormones compete for the same receptor and no synergistic effects were observed. 17β-estradiol and progesterone drastically decrease while fetal zone steroids, mainly dehydroepiandrosterone, remain persistently high within preterm infants until term. Substitution of 17β-estradiol and progesterone does not improve neurological outcomes. We investigated the influence of dehydroepiandrosterone, 17β-estradiol or dehydroepiandrosterone + 17β-estradiol treatment in C6 or OLN-93 cells on steroid receptor availability and activation of intracellular signaling molecules in hyperoxic cell culture. We sought explanations of the observed synergistic effect in preliminary study. In C6 cells, the generated signaling of dehydroepiandrosterone + 17β-estradiol treatment has no synergistic effects. The combined effect on this particular pathway does not potentiate cell survival. In OLN-93 cells, we observed significant differences in the early generated signaling of 17β-estradiol + dehydroepiandrosterone treatment to either 17β-estradiol dehydroepiandrosterone alone but never to both at the same time. The latter finding needs, therefore, further investigation to explain synergistic effects. Nevertheless, we add insight into the treatment of C6 and OLN-93 cells in hyperoxia.

The fetal zone is a compartment of the fetal adrenal cortex in humans and other higher primates. Dehydroepiandrosterone sulfate (DHEAS) is the main precursor from the fetal zone and is synthesized until the term in increasing amounts of up to 100-200 mg per day. In the placenta, DHEAS is used for biosynthesis of different estrogens, which has led to the term “feto-placental unit.” Preterm birth disconnects this unit, which results in a 100-fold drop in plasma estrogen levels compared to intrauterine levels. Notably, preterm infants’ fetal zone produces persistently high quantities of DHEAS until the time of the termed birth.

It was hypothesized that estrogen and progesterone’s substitution after preterm birth potentially protects the immature brain in case of neurological complications. In a preliminary study, 83 premature infants were randomized to supplement E2 and progesterone to maintain intrauterine concentrations. When the infants were discharged home, no effects on survival or vulnerability for intracerebral hemorrhage were observed. Five years later, no benefits were found, which underlines the need for a more detailed understanding of the role of fetal zone steroids (FZS), E2 and progesterone for the preterm brain.
In our recent work [20], we investigated the discrepancy between in vitro models and findings of the preclinical studies of Trotter et al. [17–19]. By using three different types of immature glial cells (OLN-93, C6 and PDGFRα+ cells), we found that the three most abundant FZS (DHEA, 16OH-DHEA and androstenediol) were able to protect from hyperoxia-mediated cell death in a dose-dependent manner. Furthermore, in two of the three investigated cell types (C6 and PDGFRα+ cells), additional E2 could not significantly improve the FZS-initiated neuroprotection. The investigation of the effect-mediating receptors showed that FZS could mediate protection via the estrogen receptors (ER) and the androgen receptor (AR). Because of the so-called receptor promiscuity [21], fetal zone steroids as precursors for placental estrogen synthesis are structurally related to estrogens. They thus can interact with ERs [22–24]. Furthermore, these steroids can interact with the AR [25, 26]. As a result, FZS creates a unique hormonal milieu in preterm infants, leading to continuous activation of classical ERs. Therefore, competitive effects of FSZ and E2 might be responsible for the unaltered neurological outcome in previous preliminary clinical studies [20, 27].

In general, E2 and DHEA’s effect can be mediated by two classical estrogen receptors (ERs), ER-α and ER-β, by a non-classical G protein-coupled estrogen receptor (GPER) and the AR.

The amount of functional receptors expressed by a cell is crucial for its response to hormonal ligands and can change with development, physiological and pathological status [28]. The steroid hormone receptors can be regulated by the number of ligands (autoregulation - induction or suppression) [28]. Tissue dependent autoinduction and autorepression are described for ERs and ARs. In cross-regulation, the binding of one hormone to its receptor modulates another receptor. For example, E2 can induce the progesterone receptor’s expression via cross-regulation [29]. We, therefore, investigated the effects of hyperoxia and steroid hormones on receptor density.

Different effects of hormones are mediated by different receptor type and subsequent different involved signal transduction pathways which include ERK1/2, Akt (Thr308 and Ser473) [30], AMPKa, S6RP, Bad, p70S6K, PRAS40 and GSK-3β. According to the phosphorylation status of these proteins, several pathways can be defined: (1) the PI3K/Akt/Bad signaling pathway (Akt, Bad), (2) the PI3K/Akt/mTOR signaling pathway (Akt, PRAS40, GSK-3β), and (3) the MAPK/ERK/mTOR signaling pathway (ERK1/2, p70S6K, S6RP) (see Fig. 1 for an overview). With phosphorylation of AMPKα, (4) the AMPKα/mTOR signaling pathway (AMPKα, p70S6K, S6RP) downregulates
2. Methods

2.1 Cell culture and experimental treatment

The rat-derived oligodendrocyte progenitor cell line OLN-93 [34] was cultured as described previously [35].

The rat-derived immature astroglial cell line C6 [36, 37] was purchased from the American Type Culture Collection (ATCC) as described previously.

Both cell lines were cultured to stable replication for up to ten passages after thawing in a humidified atmosphere at 37 °C and 5% CO₂ (21% O₂, normoxia; Binder, CB150).

To investigate the causes of the protective effects, concentrations were selected which exhibit a long-lasting protective effect. Only by this approach, further experiments can be conducted to investigate the underlying mechanisms. Therefore, different concentrations were used for the different substances, which exhibited a long-lasting protective effect in earlier experiments. Therefore, different concentrations were used for the different substances or cell lines. Stock solutions (1000×) of 17β-estradiol (E2; 1 μM for OLN-93 cells and 10 μM for C6 cells; Sigma-Aldrich) and dehydroepiandrosterone (DHEA; 100 nM; Sigma-Aldrich) were prepared in dimethylsulfoxide (DMSO). The DMSO was added to the control sample corresponding to the DMSO concentration in the treatment samples.

After 48 h of recovery in culture medium, OLN-93 and C6 media were exchanged to experiment media as previously described [20] containing steroids or DMSO. After 2 h of preincubation, the medium was renewed, and the cells were exposed to hyperoxia (humidified atmosphere at 37 °C with 5% CO₂ and 80% O₂; Binder, CB50) or normoxia for the indicated time.

2.2 Survival assays

C6 and OLN-93 cells were preincubated for 2 h with 1 μM and 10 μM of E2 in DMSO, respectively, and subsequently damaged by hyperoxia for 48 h. A lactate dehydrogenase (LDH) cytotoxicity assay (Abcam) was used according to the manufacturer's instructions to assess cell death in OLN-93 cells. LDH is released from damaged cells and can be measured in the supernatant. LDH oxidizes lactate and thereby produces NADH, which reacts with a red water-soluble tetrazolium to a yellow color. The color was quantified by a plate reader (Thermo Fisher, Multiscan Ex) at OD 450 nm. The OD 650 nm was measured as a reference and subtracted from OD 450 nm. Results are given as % cell death compared to DMSO-treated cells under hyperoxia.

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazotolium bromide (MTT) assay (Promega) was used according to the manufacturer's instructions to measure the viability of C6 cells. In this assay, MTT, a yellow tetrazole, is reduced to purple formazan in living cells. The conversion was measured by a plate reader at OD 595 nm and OD 650 nm as a reference and subtracted from OD 595 nm. For higher comparability among the survival assays, the viability results were processed to cell death by the formula: % cell death = 100 - (% viability - 100). Results are given as % cell death compared to DMSO-treated cells under hyperoxia.

2.3 Western blotting

To investigate the influence of steroid treatment and hyperoxia on the ER-α, ER-β, GPER and AR receptor expression, C6 and OLN-93 cells were pretreated 2 h with DMSO, E2, DHEA or E2 and DHEA and subsequently exposed to 48 h of hyperoxia. A normoxia DMSO control was included. Afterwards, proteins were isolated using TRizol Reagent (Life Technologies), followed by quantification using Roti-Quant protein quantitation assay (Carl Roth) according to each manufacturer's instruction. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad Laboratories) in a wet transfer system (all devices purchased from Bio-Rad Laboratories). The membranes were blocked 2 h in tris(hydroxymethyl)-aminoethane (Tris)-buffered saline (Carl Roth) containing 0.15% Tween 20 (Sigma-Aldrich) and 2.5% non-fat milk (Carl Roth). Afterward, membranes were incubated overnight with primary antibodies and the next day for 1 h with horseradish peroxidase-conjugated secondary antibodies. The following primary antibodies were used: anti-ER-α rabbit polyclonal antibody (1: 200; Santa Cruz), anti-ER-β rabbit polyclonal antibody (1: 1,000; Affinity BioReagent, Thermo Fisher), anti-GPER rabbit polyclonal antibody (1: 250; Abcam), anti-AR mouse monoclonal antibody (1: 200; Abcam), and anti-β-actin rabbit polyclonal antibody (1: 5,000; Sigma-Aldrich) (Table 1). Peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (H + L) secondary antibodies were purchased from Jackson ImmunoResearch (1: 5,000). Specific proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Thermo Fisher) and developed by an imaging system (Bio-Rad Laboratories, ChemiDoc XRS). Receptor proteins were quantified as a ratio to β-actin with densitometry using Image Lab software (Bio-Rad Laboratories). In ER-α, not the full-length protein (66 kDa) but the 50 kDa splice variant was investigated since it was the most dominant variant in both cell lines. By calculating the ratio...
Table 1. Antibody table.

| Name of Antibody                          | Manufacturer, catalog #, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|------------------------------------------|--------------------------------------------------------------------------|---------------------------------------------|---------------|
| Rabbit Anti-Estrogen Receptor-α (HC-20) | Santa Cruz, #sc-543                                                     | Rabbit, polyclonal                          | 1:200         |
| Rabbit Anti-Estrogen Receptor-β          | Affinity BioReagents, #PA1-311                                           | Rabbit, polyclonal                          | 1:1,000       |
| Rabbit Anti G-Protein Coupled Receptor 30| Abcam, #ab39742                                                          | Rabbit, polyclonal                          | 1:250         |
| Mouse Anti-Androgen Receptor (Clone AR-441) | Abcam, #ab9474                                                         | Mouse, monoclonal                           | 1:200         |
| Rabbit Anti-β-Actin                      | Sigma-Aldrich, #A6022                                                    | Rabbit, polyclonal                          | 1:5,000       |
| Peroxidase AffiniPure F(ab')2 Fragment Goat | Jackson ImmunoResearch, #111-036-003                                    | Goat                                        | 1:5,000       |
| Anti-Rabbit IgG (H + L)                  |                                                                           |                                             |               |
| Peroxidase AffiniPure F(ab')2 Fragment Goat | Jackson ImmunoResearch, #115-036-146                                    | Goat                                        | 1:5,000       |
| Anti-Mouse IgG (H + L)                   |                                                                           |                                             |               |

Fig. 2. Effects of E2 and DHEA co-treatment on hyperoxia-mediated cell death. Cell survival was measured (A) in C6 cells by MTT assays and (B) in OLN-93 cells by LDH assays after treatment with E2 alone (10 or 1 μM respectively), DHEA (100 nM) alone or co-treatment under hyperoxia. DMSO hyperoxia control was set to 100% to exclude inter-assay variability. Significant differences to DMSO hyperoxia control are indicated by *; significant differences between steroid treatments are indicated by #. */#, **/#, ****/### P < 0.05, ***/### P < 0.01.

2.4 Intracellular signaling array (multiplex ELISA array)

C6 and OLN-93 cells were pretreated for 2 h with DMSO, E2, DHEA, or E2 and DHEA for signaling array experiments. Cells were collected after 6 h and 24 h of hyperoxia, according to the manufacturer’s instruction. As recommended by the manufacturer, the lysis buffer was supplement with Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100×, Thermo Fisher). According to the manufacturer’s instruction, the cell lysates were quantified by Roti-Quant protein quantitation assay (Carl Roth). Afterward, cell lysates were analyzed using an intracellular signaling array kit (Cell Signaling Technology) to detect activated signaling molecules. For this actual human-specific array, 11 of 18 antibodies were species cross-reactive to rat-derived antibodies, as confirmed by the manufacturer-only 9 of those 11 antibodies delivered detectable signal.

According to the manufacturer’s instructions, cell lysates were added to the nitrocellulose-coated glass slides pre-coated with primary antibodies for 2 h. Afterward, the slides were incubated for 1 h with biotinylated antibodies, followed by a horseradish peroxidase-conjugated secondary antibody for 30 min. The slides were exposed to a substrate, and chemiluminescence was measured. Each array was processed with the “Protein Array Analyzer” macro in ImageJ (NIH) for background subtraction and grid measurement [38]. Normalization between arrays was calculated by scaling the individual intensities so that the mean intensities were the same across all compared arrays. The results show only proteins for which signal intensities were stronger than the background after 1 min of exposure. The signal intensities are given as the ratio to DMSO-treated cells, calculated for each experiment.

2.5 Statistical analysis

Statistical significance was assessed with GraphPad Prism 8.3.1 software (GraphPad Software, LLC) by Two-way ANOVA or Mixed Model depending on missing values. We corrected for multiple comparisons using Tukey’s post hoc test. Matched values were factored in as appropriate. Friedman Test was used for data of Fig. 2. The significance levels were denoted as *P < 0.05, **P < 0.01, and ***P < 0.001. A level of P < 0.05 was considered statistically significant. Data in the figures are represented as mean ± SD and result from at least four independent experiments performed on different days. Replicate wells were used and pooled for analysis. Due to the limited amount of data points per cell culture condition, some of the data must be considered explorative.
Fig. 3. Effects of steroid treatment and hyperoxia on receptor expression in C6 cells. The expression of (A) 50 kDa splice variant of ER-α, (B) ER-β, (C) GPER and (D) AR in C6 cells were analyzed by western blot after treatment with E2 alone (10 μM), DHEA (100 nM) alone or co-treatment. Data were normalized to normoxia DMSO control (set to 100%). Significant differences between hyperoxia DMSO control and treatments are indicated by * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$.

Fig. 4. Effects of steroid treatment and hyperoxia on receptor expression in OLN-93 cells. The expression of (A) 50 kDa splice variant of ER-α, (B) ER-β, (C) GPER and (D) AR in OLN-93 cells was analyzed by western blot after treatment with E2 alone (1 μM), DHEA (100 nM) alone or co-treatment. Data were normalized to normoxia DMSO control (set to 100%). Significant differences between hyperoxia DMSO control and treatments are indicated by * $P \leq 0.05$, ** $P \leq 0.01$; *** $P \leq 0.001$. 
Fig. 5. Intracellular signaling among hyperoxia and treatments in C6 cells. To explore intracellular signaling, a signaling array was used. After 2 h treatment with DMSO, E2, DHEA, or DHEA/E2 co-treatment, cells were exposed to 6 h and 24 h of hyperoxia. C6 cells were treated with DMSO, 10 μM E2, 100 nM DHEA, or DHEA and E2 combined and afterward exposed to (A) 6 h and (B) 24 h hyperoxia. Significant differences from hyperoxia/DMSO control, set to 1.0, are indicated by # \( P \leq 0.05 \). Significant differences between steroid treatments are indicated by * \( P \leq 0.05 \). White bars represent E2-treated cells, light grey bars represent DHEA-treated cells, and dark gray bars represent DHEA/E2-co-treated cells.

3. Results

3.1 Effects of E2 and DHEA co-treatment on hyperoxia-mediated cell death

Under hyperoxia, the effect of treatment on cell survival with DHEA alone, co-treatment with E2 or E2 alone was compared to DMSO control (set to 100%) (Fig. 2A, B). While all hormone conditions reduced cell death significantly in both cell types, co-treatment significantly increased cell survival compared to DHEA or E2 single treatment in OLN-93 cells (\( P \leq 0.001 \); Fig. 2B).

3.2 Effects of steroid treatment and hyperoxia on the receptor expression

To investigate the influence of steroid treatment and hyperoxia on the steroid receptor availability, the expression of ER-\( \alpha \), ER-\( \beta \), GPER and AR was analyzed. For these experiments, C6 and OLN-93 cells were pretreated for 2 h with DMSO, E2, DHEA, or E2 and DHEA and afterward subjected to 48 h of hyperoxia.

In C6 cells, neither steroid treatment nor hyperoxia led to significant ER-\( \alpha \) or \( \beta \) or GPER expression changes compared to DMSO hyperoxia control (Fig. 3A, B, C). Treatment with E2 led to a significant increase of AR compared to DHEA (\( P \leq 0.05 \)) (Fig. 3D).

In OLN-93 cells, treatment with DHEA plus E2 led to a significant increase of ER-\( \alpha \) compared to DMSO under hyperoxia (\( P \leq 0.01 \)). DHEA (\( P \leq 0.01 \)) and its combination with E2 (\( P \leq 0.001 \)) increased ER-\( \alpha \) significantly compared to E2 alone (Fig. 4A). The relative quantification of ER-\( \beta \) led to similar results (Fig. 4B).

There were no significant differences for GPER or AR observed under hyperoxia (Fig. 4C, D).

3.3 Effects of steroid treatment on intracellular signaling molecules

In C6 cells, ERK1/2 (Thr202/Tyr204), Akt (Thr308 and Ser473), S6 ribosomal protein (Ser235/236; S6RP), Bad (Ser112), ribosomal protein S6 kinase (Thr389; p70S6K), proline-rich Akt/PKB substrate 40 kDa (Thr246; PRAS40), and glycogen synthase kinase-3\( \beta \) (Ser9; GSK-3\( \beta \)) were detectable while Stat3 (Tyr705) or p38 (Thr180/Tyr182) were not (Fig. 5A, B).

After 6 h of hyperoxia, Akt (Ser473) was significantly downregulated, whereas GSK-3\( \beta \) was significantly upregulated in DHEA plus E2-treated cells compared to DMSO control (Fig. 5A). The activation of Akt seemed to be transient. After 24 h of hyperoxia, there were no significant differences in E2- and DHEA-treated cells (Fig. 5B). Under DHEA + E2 co-treatment, Bad, PRAS40, and GSK-3\( \beta \) were significantly upregulated compared to DMSO control. There were no differences between the different steroid treatments (Fig. 5A, B).

In OLN-93 cells, the signaling mentioned above molecules and AMP-activated protein kinase \( \alpha \) (Thr172; AMPK\( \alpha \)) were detectable. Again, no distinct activation of Stat3 or p38 was measured (Fig. 6A, B).

After 6 h of hyperoxia, there was a significant downregulation of GSK-3\( \beta \) under DHEA treatment (Fig. 6A). Of interest, there was a significant difference between DHEA single and its combination treatment with E2 in phosphorylation...
of Akt (Thr308). The activation of Akt in E2 and DHEA/E2 co-treated cells seems to be transient as in the C6 cells because there was a significant upregulation of the Akt pathway (Fig. 6A).

After 24 h of hyperoxia, no significant differences were observed (Fig. 6B).

4. Discussion

During pregnancy, FZS is synthesized by the fetus and serve as precursors for estrogen synthesis in the placenta [12]. It was previously shown that FZS synthesis in preterm infants is persistently high until term [15, 16]. Our recent work showed that FZS activates classical ERs, which lead to protective properties against hyperoxia-induced cell death [20]. The experimental hyperoxia model was applied to mimic the oxygen supplementation used in neonatal intensive care in resuscitation and treatment of neonatal lung disease. Due to a dramatic rise in oxygen tissue tension, premature infants are subjected much earlier to relative hyperoxia than intrauterine conditions. However, in C6 cells under hyperoxia, co-treatment of DHEA and E2 showed no synergistic effects while both hormones competed for the same receptor [20]. In contrast, in OLN-93 cells under hyperoxia, co-treatment led to synergistic effects while E2 and DHEA mediated their effects via different receptors as published earlier by our group [20]. To further elucidate the circumstances of synergism, we now aimed to investigate the effects of DHEA and E2 alone and their combination on the receptor availability and the induced signaling pathways.

4.1 Effects of hyperoxia and steroid hormones on receptor densities

The effects of hyperoxia and steroid hormones on receptor density have been investigated in only two studies to date. In one study, astrocytes were shown to downregulate the amount of progesterone receptor by hyperoxia [39]. The other study showed in cultured smooth muscle cells of the respiratory tract that hyperoxia (with 50% O2) does not change the receptor density of ER-α and ER-β [40] in human fetuses.

Hyperoxia induced significant changes in receptor expression compared to DMSO control in OLN-93 cells. The effects on the C6 cells were the only minor. Since a similar trend in regulation could be observed, the adverse finding could be based on the limited number of experiments.

Since full-length ER-α was hardly detectable in both cell types and there was a relatively large amount of a ~50 kDa splice variant of ER-α, this variant was quantified. The mRNA of ER-α can undergo alternative splicing, whereby one or more exons are deleted [41]. In the 50 kDa band for ER-α examined here, exon 7 is depleted [42]. Not much is known about this splice variant so far. However, it does not seem to modulate gene expression [43]. The function of this splice variant in receptor-mediated protection should be the subject of future investigations.

In C6 cells, E2 seems to induce AR via cross-regulation under hyperoxia. The induction of AR via E2 has already been described for other tissues [44]. However, it remains to be clarified whether the effect of DHEA is an amplification of autoinduction and/or a cross-regulation since DHEA can bind to classical ERs and AR.

In OLN-93 cells, ER-α and -β were upregulated under the pathological situation. However, this effect was reversed for all three receptors by treatment with E2 by autorepression. This autorepression by E2 was previously also found in other estrogen-dependent tumor cells [45].

4.2 The phosphorylation status of intracellular signaling molecules

Receptor-mediated survival can be initiated via genomic and non-genomic pathways. The activation of signal transduction pathways can be temporary (transient activation) or persistent (permanent activation). Transient signals can be activated just a few minutes after adding factors and can disappear after minutes or hours [46, 47]. E2 and DHEA can induce non-genomic neuroprotective signaling pathways via ERK1/2 and Akt [8, 48, 49].

It was previously shown that PI3K/Akt/Bad signaling mediates protection of E2 against oxidative stress-induced apoptosis in C6 cells [50], and treatment with DHEA prevents cell death via PI3K/Akt/mTOR signaling in newborn neurons [49]. Even without sustained upregulation of Akt itself, we found downstream signaling proteins of the PI3K/Akt/Bad and mTOR pathways significantly upregulated in C6 cells after 24 h of hyperoxia in the co-treatment group. Of interest, it was previously reported that induction of cell death could induce ERK1/2 phosphorylation in astrocytes [51, 52], and E2 can reverse hyperoxia-induced phosphorylation of ERK1/2 in astrocytes [53].

Although it was shown that E2 activates ERK1/2 and Akt signaling to prevent cell death in oligodendrocyte precursor cells [8], we only found downregulation of GSK-3β under DHEA treatment after 6 h in OLN-93 cells. This might be due to differences in cell culture, experimental setting or even the used oligodendrocyte cell type.

5. Conclusions

Our findings show that the generated signaling of E2 + DHEA treatment in C6 cells has no synergistic effects. Thus, although the same signaling route is addressed, the combined effect on this particular pathway does not potentiate cell survival. On the other hand, we observed improved cell survival after E2 + DHEA treatment in OLN-93 cells. Here we found significant differences in the early generated signaling of E2 or DHEA treatment compared to E2 + DHEA cells but never both at the same time.

The observed changes in the receptor densities do not affect the occurrence of synergism. Furthermore, two neuroprotective treatments that initiate the same signaling pathway might not cause synergism since the full signaling capacity was already reached by one of the stimulants alone. If two neuroprotective treatments initiate different or differently timed signaling, synergism might occur more quickly. Synergistic effects of DHEA and E2 as shown in OLN-93 cells
Fig. 6. Intracellular signaling among hyperoxia and treatments in OLN-93 cells. To explore intracellular signaling, a signaling array was used. After 2 h treatment with DMSO, E2, DHEA, or DHEA/E2 co-treatment, cells were exposed to 6 h and 24 h of hyperoxia. OLN-93 cells were treated with DMSO, 1 µM E2, 100 nM DHEA, or DHEA and E2 combined and afterward exposed to (A) 6 h and (B) 24 h hyperoxia. Significant differences from hyperoxia/DMSO control, set to 1.0, are indicated by # $P \leq 0.05$. Significant differences between steroid treatments are indicated by * $P \leq 0.05$. White bars represent E2-treated cells, light grey bars represent DHEA-treated cells, and dark gray bars represent DHEA/E2-co-treated cells.

are not likely to occur in preterm infants due to the receptor affinities of human receptors that will not lead to predominant AR activation by FZS (e.g., DHEA: ER-β $K_d \approx 0.5 \mu M$; AR $K_d \approx 1.1 \mu M$) [22, 25]. Nevertheless, the OLN-93 cells can give hints for the development of future treatment strategies in preterm infants. For this purpose, the intracellular processes initiated by the respective treatments should be investigated in detail. Therefore, a treatment that induces intracellular signaling distinct from the endogenous FZS-initiated signaling processes might support the clinical outcome after preterm birth in a synergistic way. This support might also include activation of the same signaling family but with other specific characteristics.

Auto- and cross-regulation are essential mechanisms for amplifying hormone signals, regulating hormone activities by negative feedback mechanisms, and coordinating hormone effects in a quick and tissue-specific manner [28]. In general, autorepression’s most crucial function is to restore homeostasis by downregulating the receptor as the hormones increase [28]. This effect could not be found in either of the cell types studied within the observation period. Future work would require investigating how the severe deficiency of estrogens and progesterone, together with the large number of adrenal androgens (fetal zone steroids), affects the receptor composition in the premature infant after birth and in the long term.

Abbreviations

16OH-DHEA, 16α-hydroxy-dehydroepiandrosterone; AMPKα, AMP-activated protein kinase α; AR, androgen receptor; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; ER, estrogen receptor; ERK1/2, extracellular signal-regulated kinase; FZS, fetal zone steroids; GPER, G protein-coupled estrogen receptor; GSK-3β, glycogen synthase kinase 3β; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P13, phosphatidylinositol 3; P13K, phosphatidylinositol 3 kinase; PRAS40, proline-rich Akt/PKB substrate 40kDa; p70S6K, ribosomal protein S6 kinase; S6RP, S6 ribosomal protein.
Author contributions
SH did the investigation, formal analysis and writing (original draft preparation). DS und CZ did investigation and writing (review & editing). JR did the investigation, formal analysis and writing (review & editing). BR did supervision and writing (review & editing). AV and MH did conceptualization, supervision and writing (review & editing).

Ethics approval and consent to participate
Not applicable.

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Conflict of interest
The authors declare that they have no competing interests. Given his role as the Review Board Member of JIN, Prof. Matthias Heckmann had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

Consent for publication
Not applicable.

Availability of data and materials
Raw data is available from the authors upon reasonable request.

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